

**Imperial College
London**

**Dissecting mechanisms of endocardial
cell volume regulation
in zebrafish cardiac valve morphogenesis**

PhD Thesis

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Imperial College London, for the Award of the degree of PhD in Bioengineering
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Statement of Originality

I hereby declare that this thesis is the result of my own work under the supervision of Dr Julien Vermot. The work was performed in the Department of Bioengineering at Imperial College London. All published and unpublished materials used in the thesis have been given full acknowledgement. This work has not been previously submitted, in whole or in part, to any other academic institution for a degree, diploma, or any other qualification.

Christina Vagena-Pantoula

February 2024, London

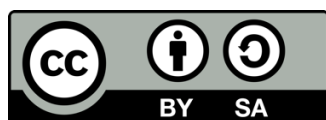
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Abstract

Amongst the mechanisms involved in tissue development, cell volume regulation emerges as an important modulator of morphogenesis. In zebrafish, endocardial cell volume decrease is pivotal for heart valve formation. However, the cellular and molecular mechanisms underlying endocardial cell volume changes remain unknown.

Here, I demonstrate that the interplay of the mechanosensitive ion channel Piezo1, the receptor Notch1b and an aquaporin (Aqp) water channel controls endocardial cell volume reduction. I show that Aqp8a.1 is required for endocardial cell volume regulation, and *aqp8a.1* mutant larvae display heart valve defects, altered cell polarity and absence of F-actin remodelling. Furthermore, I demonstrate that *aqp8a.1* expression is regulated by Notch signalling. Mechanistically, overexpression of Piezo1 using the genetically encoded Piezo1 biosensor GenEPi leads to downregulation of *notch1b* and subsequently *aqp8a.1*. At the protein level, the Ca²⁺-binding calmodulin directly binds to the Aqp8a.1 carboxyl terminus, driving Aqp8a.1 localisation in response to mechanical stimuli. In parallel with these mechanisms, F-actin remodelling through the actin crosslinking protein Marcksl1 arises as an essential event for AVC EdC cell volume reduction.

Altogether, this work identifies new effectors of endocardial cell volume changes and heart morphogenesis, thereby increasing our fundamental knowledge on early organogenesis.

Contribution Statement

Due to the considerable collaboration involved in this research, the contributions of the researchers who supported the work presented in this thesis are recognised below:

Dr Julien Vermot supervised the project. Dr Julien Vermot and Christina Vagena-Pantoula conceptualised this work and the related experiments. Christina Vagena-Pantoula designed, planned, and conducted the experiments, as well as performed the data analysis of the acquired datasets, unless otherwise mentioned below.

- Dr Konstantinos Kalyviotis and Dr Periklis (Laki) Pantazis provided the *Tg(hsp70l:GenEPi)* line. Dr Konstantinos Kalyviotis conducted the site-directed mutagenesis experiments.
- Antoine Sanchez performed the endocardial nuclei segmentation and volume measurement, under the supervision of Dr Julien Vermot.
- Dr Igor Kondrychyn and Dr Li-Kun Phng provided the plasmids *fli1ep:aqp8a.1-mEmerald*, *fli1ep:aqp8a.1-mCherry* and *fli1ep:aqp1a.1-mEmerald*, and generated the *aqp8a.1^{rk29};aqp1a.1^{rk28}* and *marcksl1^{ark23};marcksl1b^{ark24}* mutants. Dr Igor Kondrychyn provided datasets of *aqp8a.1* and *aqp1a.1* mutants in a *fli1:H2B-EGFP* background.
- Dr Thomas Juan and Prof Didier Stainier provided the constructs for the generation of the *piezo1* and *piezo2a in situ* probes, as well as the *zcamk2g1^{T287D}-pCS2+*. Thomas Juan generated the *piezo1^{bns340}* and *piezo2a^{bns367}* mutants and provided fixed *piezo1^{bns340}* and *piezo2a^{bns367}* mutant embryos.

Related Work

Peer Review Publications

**Equal contribution

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Abbreviations

3D	three-dimensional
ALPM	anterior lateral plate mesoderm
Aqp1a.1	Aquaporin 1a.1
Aqp8a.1	Aquaporin 8a.1
ATP	adenosine-triphosphate
AV	atrioventricular
AVC	atrioventricular canal
bp	base pair
CaM	calmodulin
cAMP	cyclic adenosine monophosphate, an intracellular signalling molecule
cDNA	complementary DNA
CHD	congenital heart disorder
CJ	cardiac jelly
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9
DAPI	diamino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpf	days post-fertilisation
ECM	extracellular matrix
EdC	endocardial cell
ER	endoplasmic reticulum
FP	fluorescent protein
GCaMP	genetically encoded green calcium indicator, based on GFP,

	calmodulin and myosin light chain kinase M13 peptide
GFP	green fluorescent protein
hpf	hours post-fertilisation
hsp70-l	heat shock protein 70-like zebrafish promoter
MO	morpholino antisense oligonucleotide
MR	mitral valve regurgitation
mRNA	messenger RNA
OFT	outflow tract
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
SL	semiluminar
TALEN	transcription activator-like effector nuclease
VIC	valve interstitial cell
WT	wild type, the phenotype found in nature, generated by the unmodified version of a gene
ZFN	zinc finger nuclease

Chapter 1. Introduction

1.1 The Cardiovascular System

The cardiovascular system, responsible for ensuring adequate blood circulation throughout the body, is essential for maintaining life. It is comprised of the heart, arteries, veins, and capillaries, and responds to various stimuli to regulate the blood supply. This intricate system supports the delivery of nutrients and oxygen to cells, as well as the removal of waste products, rendering critical organismal functions sustainable (Pittman, 2011, Humphrey and McCulloch, 2003, Chaudhry et al., 2023). Arteries serve a specific role in carrying oxygenated blood away from the heart to various tissues and organs. As they progress towards individual cells, they gradually decrease in size, eventually transforming into capillaries. This reduction in diameter considerably lowers the blood flow rate, facilitating the efficient extraction of nutrients by cells and exchange of metabolic waste (**Figure 1**). Deoxygenated blood is then conveyed back to the heart via the network of veins (Titchenal et al., 2023, Fleischer et al., 2020, Tucker et al., 2023).

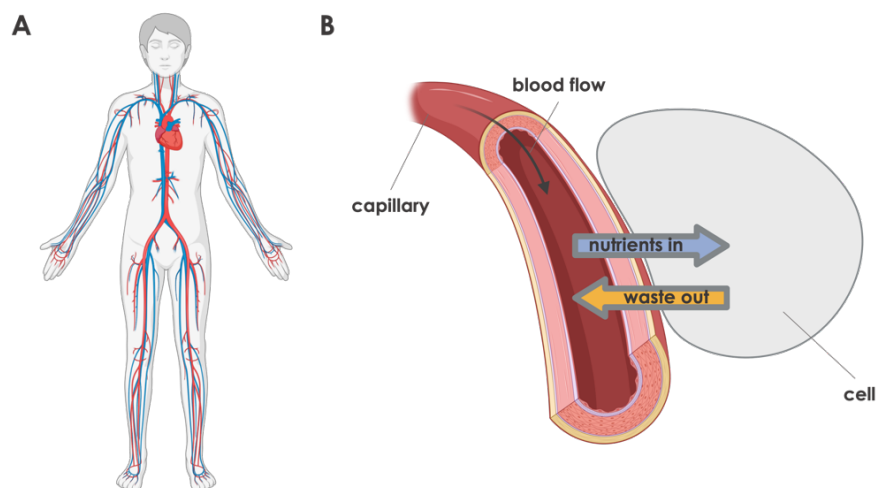


Figure 1 | The cardiovascular system in human. (A) The heart, arteries (depicted in red), veins (depicted in blue), and capillaries form the cardiovascular system, enabling continuous blood flow throughout the body. (B) The cardiovascular system facilitates the exchange of oxygen/ nutrients and waste in all cells. (Adapted from (Titchenal et al., 2023) and created with Biorender.com).

1.2 Physiological function and anatomy of the heart

The heart serves as the primary organ within the cardiovascular system and functions as a pump, propelling blood through the vessels (Chaudhry et al., 2023, Rehman and Rehman, 2023, O'Donnell and Yutzey, 2020). In mammals, the heart is comprised of four chambers, two upper atria and two lower ventricles. The atrioventricular (AV) valves, namely the tricuspid and mitral valves, distinguish the atria from the ventricles, whereas the aortic and pulmonary semilunar (SL) valves separate the ventricles from the great arteries (**Figure 2**). AV valves are composed of leaflets, while SL valves have cusps. The cardiac valves ensure the unidirectional flow of the blood within the heart (Hinton and Yutzey, 2011).

The heart provides blood flow to the pulmonary and systemic circuits (Rehman and Rehman, 2023, Buckberg et al., 2018). Systemic circulation ensures the distribution of oxygenated blood and nutrients to the entire body, while pulmonary circulation facilitates blood oxygenation. The process begins with the contraction of the left ventricle, resulting in the ejection of oxygen-rich blood from the heart muscle to the body through the aorta. Subsequently, oxygen-depleted blood from the body enters the right atrium via the superior and inferior vena cavae, along with deoxygenated blood from the heart itself through the coronary sinus. The blood then progresses through the tricuspid valve to reach the right ventricle, from where it flows to the lungs for oxygenation through the pulmonary artery. Once reoxygenated, the blood is transported to the left atrium via the pulmonary vein. Ultimately, it is transferred to the left ventricle by either passive flow or active pumping, through the mitral valve, initiating a new cycle (**Figure 2**). This complex mechanism enables the continuous flow of blood throughout the body, crucial for sustaining life (Chaudhry et al., 2023, Rehman and Rehman, 2023).

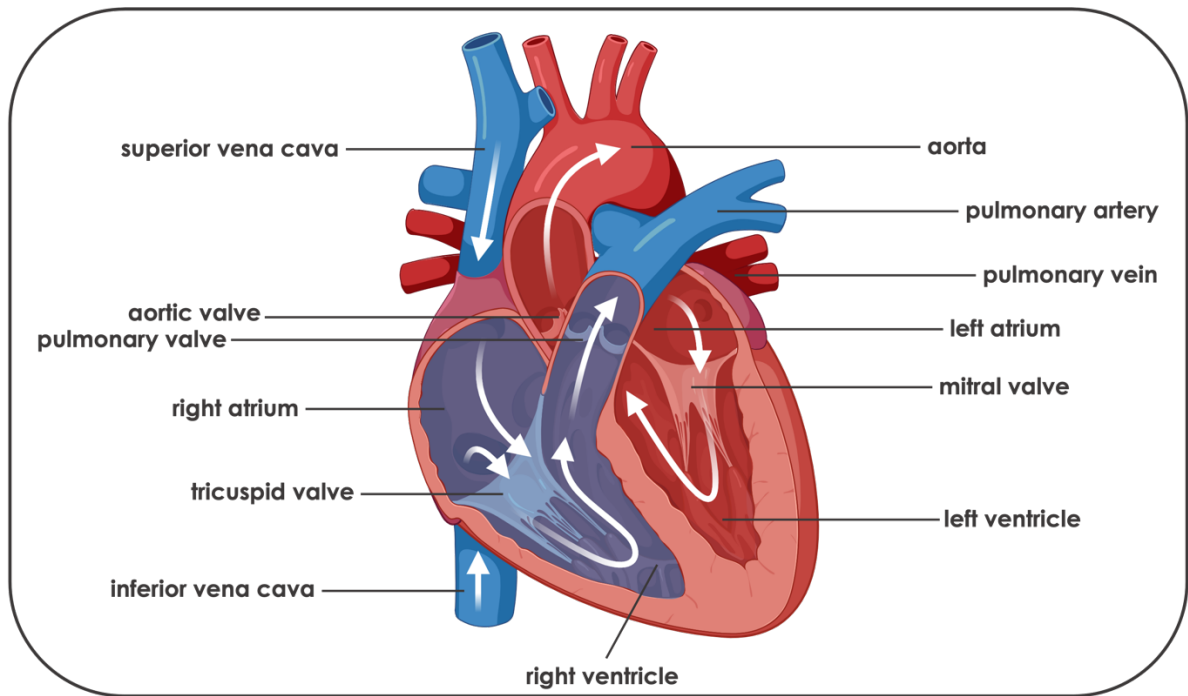


Figure 2 | The human heart. The human heart is four-chambered and consists of two ventricles and two atria. The oxygen-rich blood exits the heart and the left ventricle through the aorta, while the deoxygenated blood returns to the heart muscle and the right atrium via the superior and inferior vena cavae. White arrows indicate the direction of blood flow. (Created with Biorender.com).

The vertebrate heart is composed of three tissue layers: the endocardium, the myocardium, and the epicardium. Each layer contributes to the overall structure and function of the heart, ensuring its coordinated and efficient performance (Arackal and Alsayouri, 2023).

The epicardium constitutes the outermost layer, acting as a shield, protecting the heart muscle from external stress and potential damage. Formed by evolutionary conserved mesothelial cells, it is involved in diverse developmental and physiological processes crucial for the heart's operation. Notably, the epicardium participates in foetal cardiac development, coronary vessels patterning, and heart regeneration (Quijada et al., 2020).

The myocardium is the middle layer of the heart wall. It is comprised of cardiomyocytes, contractile cells that enable the contraction of the heart and,

therefore, the pumping of blood throughout the cardiovascular system. Remarkably, the myocardium contains highly specialised cells, namely pacemaker cells, with an intrinsic ability to generate electrical impulses. This capability induces a timely contraction and ensures a regular heartbeat (Wei et al., 2023, Kennedy et al., 2016).

The innermost tissue layer of the heart consists of a monolayer of specialised endothelial cells, termed the endocardium. This cell layer lines the chambers of the heart, including the cardiac valves, and is directly exposed to the flow of blood (Haack and Abdelilah-Seyfried, 2016, Dye and Lincoln, 2020, Tian and Morrissey, 2012). Functioning as a physical barrier, the endocardium safeguards the inner cardiac muscle tissue against haemodynamic forces (Haack and Abdelilah-Seyfried, 2016, Dye and Lincoln, 2020). The endocardial cell layer acts as both a sensor and transducer of signals generated by blood flow mechanical forces. This mechanotransduction process is essential for the formation of cardiac structures, including the heart valves (Chow et al., 2022, Fukui et al., 2021, Vignes et al., 2022, Dye and Lincoln, 2020, Haack and Abdelilah-Seyfried, 2016, Vermot et al., 2009, Steed et al., 2016a, Steed et al., 2016b, Boselli et al., 2017, Heckel et al., 2015, Duchemin et al., 2019, Goddard et al., 2017). Additionally, the endocardium serves as an angiocrine signalling hub actively engaged in myocardial cell morphogenesis (Bussmann et al., 2007, Holtzman et al., 2007, Schumacher et al., 2013, Stainier et al., 1995) and regeneration (Choi et al., 2013, Sanz-Morejón and Mercader, 2020).

Overall, the three tissue layers forming the heart wall serve distinct functions, collectively required for the optimal coordination and operation of the heart.

1.3 Congenital heart disorders

Congenital heart disorders, commonly referred to as congenital heart defects (CHDs), are structural anomalies of the heart or intrathoracic great vessels. They occur during embryonic development and potentially cause functional impairment (Mitchell et al., 1971, Hoffman and Kaplan, 2002). CHDs are the most prevalent congenital abnormalities, with an estimated frequency of 8 to 9 in 1000 live births, and they represent the leading cause of morbidity and mortality globally (Suluba et al., 2020, Ossa Galvis et al., 2023).

1.3.1 Congenital heart valve defects

Valvular abnormalities are present in the majority of congenital cardiac malformations and account for 10% of all CHDs. While they primarily arise during foetal development, they can also emerge due to inflammatory diseases, degeneration, or infection. Heart valve defects can result in cardiac malfunction, accompanied by haemodynamic consequences (Saef and Ghobrial, 2021, LaHaye et al., 2014, Hollenberg, 2017).

The prevalent types of valvular CHDs include stenosis and regurgitation, which can affect any of the four heart valves: tricuspid, pulmonary, mitral, and aortic. Stenosis refers to a condition in which the orifice area of the valve is decreased due to the thickening, stiffening or fusion of the valve leaflets/ cusps (Rana, 2022, LaHaye et al., 2014, Saef and Ghobrial, 2021). As a result, blood flow is restricted through the valve (Baumgartner et al., 2009) and the subsequent pressure overload can lead to hypertrophy (Rader et al., 2015). Aortic stenosis ranks as the second most common valve anomaly (**Figure 3A**) (Hollenberg, 2017).

Regurgitation defects involve inadequate closure of the valve, resulting in retrograde flow or leakage of blood (**Figure 3B**) (Apostolidou et al., 2017). Mitral valve regurgitation (MR) is the most prevalent valvular heart disease, characterised by reverse blood flow from the left ventricle into the left atrium (El Sabbagh et al., 2018). It can emerge from defects either in the valve leaflets themselves or in any structure comprising the mitral apparatus. MR leads to left ventricular overload, as the increased blood volume within the left atrium causes elevated preload in the left ventricle during diastole. Over time, this overload contributes to ventricular dilatation, impaired contractility, and thus decreased ejection fraction, diminishing the efficacy of the heart's pumping mechanism (Maganti et al., 2010, Apostolidou et al., 2017, Enriquez-Sarano et al., 2005).

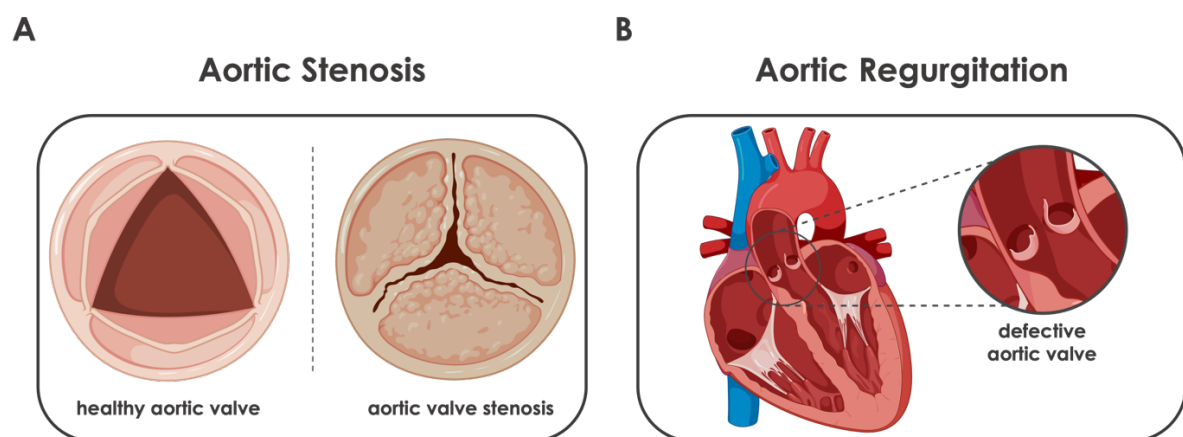


Figure 3 | Aortic stenosis and aortic regurgitation. (A) Aortic stenosis is marked by a decreased valve orifice area, constraining the flow of blood through the valve, while (B) in aortic regurgitation, the valve cusps do not close sufficiently, leading to retrograde blood flow. (Created with Biorender.com).

The pathogenesis of congenital heart and valve diseases remains unclear, yet genetic and environmental factors may contribute to their occurrence. Unfortunately, the ensuing damage to the heart is irreversible owing to its inherent lack of regenerative capacity, often necessitating surgical procedures (Suluba et al., 2020, Levine et al., 2015). Therefore, it becomes imperative to enhance our knowledge of early heart development in order to understand the origins of CHDs and advance therapeutic approaches and strategies.

1.4 The zebrafish as a model for cardiac morphogenetic studies

Since its introduction in the late 1970s (Streisinger et al., 1981), the zebrafish (*Danio rerio*) has emerged as a powerful model system for unravelling the complexities of cardiac development and congenital heart disorders.

Amongst the plethora of the advantages it offers, the optical transparency of zebrafish embryos enables non-invasive monitoring of the developing heart (**Figure 4**). Coupled with the external fertilisation of zebrafish eggs, allowing for the manipulation of embryos from the one-cell stage, this feature is invaluable for gaining insights into the temporal and spatial aspects of early developmental procedures (Hoo et al., 2016, Bakkers, 2011, Briggs, 2002, Veldman and Lin, 2008).

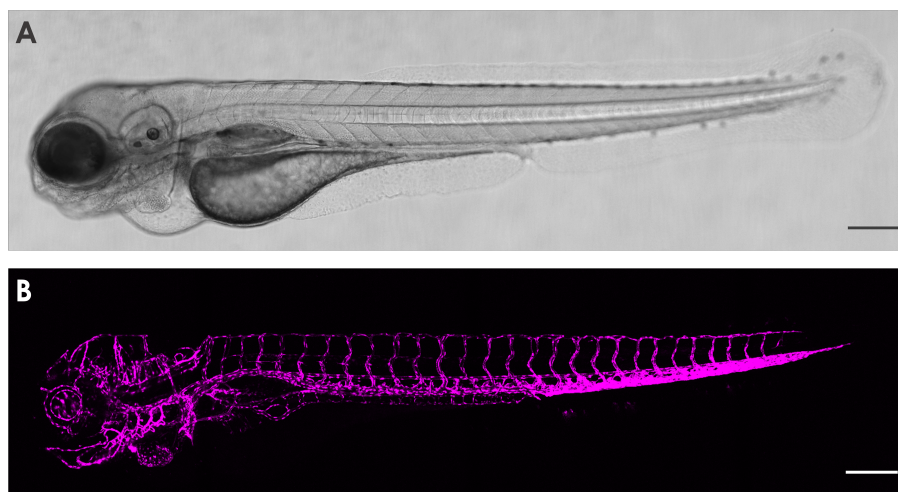


Figure 4 | The zebrafish larva and its vasculature. (A) Brightfield image of a transparent zebrafish larva at 98 hours post-fertilisation (hpf). (B) Confocal image of the zebrafish vasculature at 98 hpf, using the *Tg(kdrl:NLS-mCherry)* line. Scale bars: 200 µm.

Studying the developmental processes that govern the formation of the heart has been challenging, since most vertebrates, including mouse and chick embryos, rely on a functional cardiovascular system and do not survive early heart defects. The zebrafish stands out as a notable exception that enables the examination of severe heart phenotypes. This is due to its small size, which facilitates a sufficient oxygen

supply through passive diffusion, enabling survival even in the absence of heart contraction and active blood circulation (Tu and Chi, 2012, Stainier, 2001, Brown et al., 2016).

In addition to this unique property, the zebrafish heart shares many characteristics with the amniote heart, indicating a common evolutionary origin (Staudt and Stainier, 2012). The complete sequencing of the zebrafish genome has revealed that 71.4% of human genes have at least one zebrafish orthologue, underscoring the high genetic similarity (Howe et al., 2013).

As a result, the zebrafish has been instrumental in advancing our understanding of vertebrate genes implicated in cardiac development. Large-scale mutagenesis screens have resulted in the identification of dozens of zebrafish mutants with genetic alterations that impede the development or functionality of the heart and vasculature. The examination of these mutants has uncovered molecular mechanisms involved in morphogenetic and developmental processes (**Figure 5**) (Stainier et al., 1996).

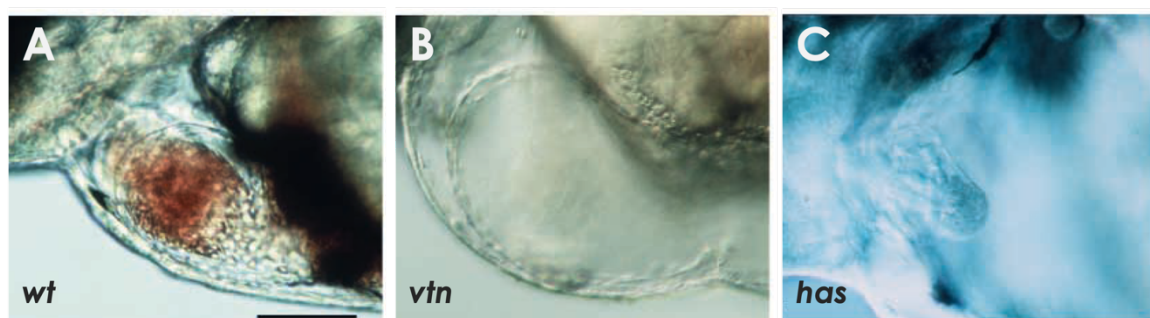


Figure 5 | Resulting cardiac phenotypes of a large-scale mutagenesis screen. Nomarski views of living embryos: (A) wild-type heart, (B) enlarged heart of a *vtn*^{m201} mutant, (C) smaller heart of a *has*^{m129} mutant. *vtn*: valentine, *has*: heart and soul. Scale bar: 100 μ m. (Adapted from (Stainier et al., 1996)).

Complementary to forward genetic approaches, various tools can facilitate alteration of specific genes of interest. Morpholino antisense oligonucleotides

(MOs) can provide a rapid means of disrupting genes of interest (Eisen and Smith, 2008), however they often lead to nonspecific effects, especially within the cardiovascular system. Advancements in gene-editing technologies, including clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR/Cas9) (Li et al., 2016), transcription activator-like effector nucleases (TALENs) (Huang et al., 2011, Sander et al., 2011) and zinc finger nucleases (ZFNs) (Egger, 2008), have allowed specific gene modifications and do not suffer from off-target effects compared to morpholinos, contributing to the generation of new knowledge in the field.

Remarkably, the zebrafish also exhibits a distinctive ability for cardiac structural and functional regeneration. Unlike mammals, including humans, the zebrafish heart can effectively regenerate in the event of injury or damage (González-Rosa et al., 2011, Chablais et al., 2011, Jopling et al., 2010, Kikuchi et al., 2010, Schnabel et al., 2011, Wang et al., 2011, Poss, 2007, Sanz-Morejón and Mercader, 2020, Marques et al., 2019). As the regenerative process can activate the expression of embryonic cardiogenesis genes (Kikuchi et al., 2010), decoding developmental procedures may shed light on the high regenerative capacity of zebrafish.

Overall, the zebrafish is widely recognised as an exemplary model organism, offering significant advantages for studying the development of the cardiovascular system, and particularly the heart.

1.5 The zebrafish heart

The cardiac structure of the zebrafish is less complex compared to that of mammals. The zebrafish heart is two-chambered, consisting of a ventricle and an atrium, separated by the atrioventricular canal (AVC) (Bartman et al., 2004, Beis et al., 2005). Cardiac valves at the atrioventricular and outflow tract (OFT) junctions emerge from the activity of specific endocardial cells (EdCs) and facilitate forward blood flow throughout the cardiac cycle (O'Donnell and Yutzey, 2020, Henderson et al., 2020). The zebrafish heart is composed of three tissue layers: the endocardium, composed of specialised endothelial cells, the intermediate contractile myocardium, and the outermost epicardium (**Figure 6**) (Brown et al., 2016).

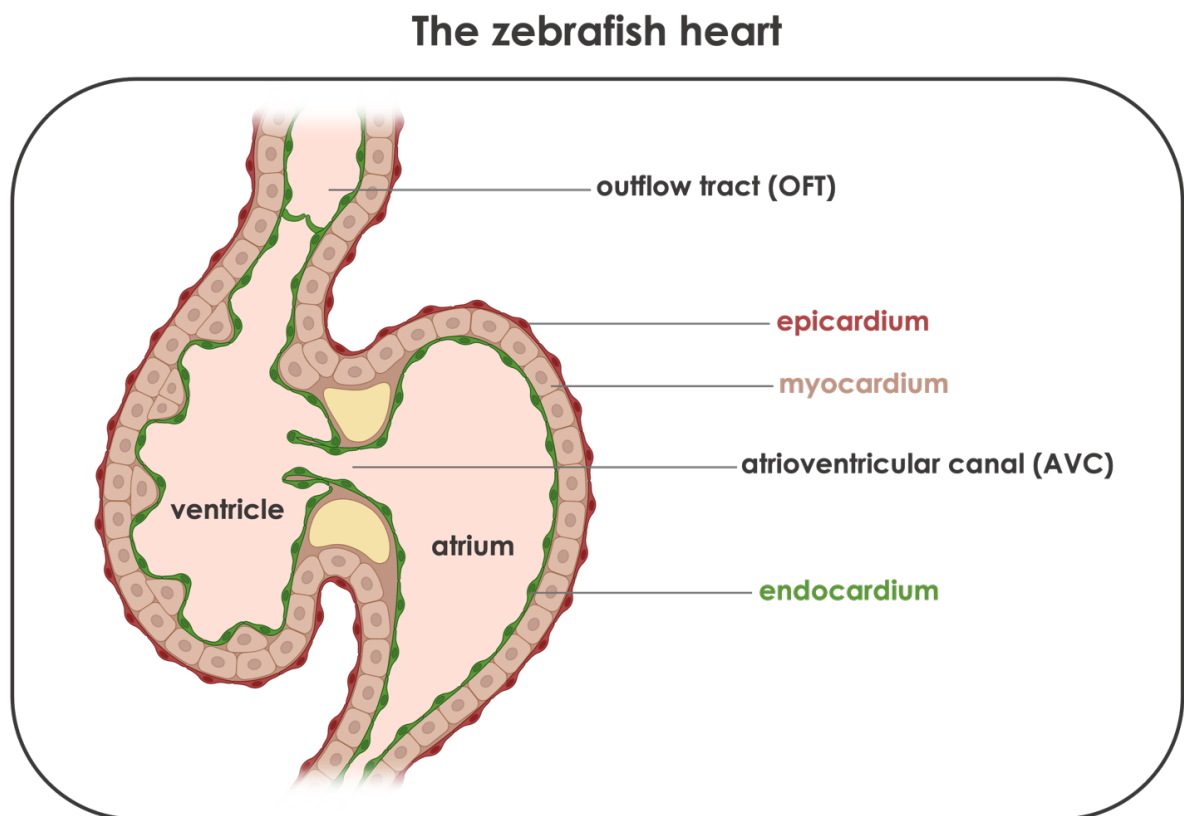


Figure 6 | The zebrafish heart. Schematic illustrating a cross-sectional view of the zebrafish heart at 5 days post-fertilisation (dpf). (Adapted from (Brown et al., 2016) and created with Biorender.com).

1.5.1 Heart development in zebrafish

In vertebrate embryogenesis, the heart is the first organ to take shape and become functional. During early development, the embryonic zebrafish heart comprises only two tissue layers: the endocardium and the myocardium, with the cardiac jelly (CJ) or extracellular matrix (ECM) lying in between. Cardiac progenitors, encompassing both myocardial and endocardial cells, can be identified as early as the blastula stage (Keegan et al., 2004).

Prior to the onset of gastrulation, at 5 hours post-fertilisation (hpf), myocardial and endocardial progenitors are located in the ventral and lateral regions on both sides of the embryo (Keegan et al., 2004, Stainier et al., 1993, Lee et al., 1994). Ventricular myocardial progenitors are positioned more dorsally than their atrial counterparts, while endocardial progenitors are not organised by chamber (Keegan et al., 2004).

By the 6-9 somite stages, at around 12 hpf, myocardial progenitors converge towards the posterior half of the anterior lateral plate mesoderm (ALPM), with atrial precursors being enriched more laterally compared to ventricular precursors (Yelon et al., 1999). Endocardial progenitors are found anteriorly in the ALPM, more medially than the myocardial precursors (**Figure 7A**) (Harvey and Rosenthal, 1999).

By 19 hpf and the 20-somite stage, endocardial and myocardial progenitor cells move towards the midline and fuse to form the cardiac cone or cardiac disk, which begins its first contractions approximately at 21-22 hpf (**Figure 7B**) (Stainier, 2001, Brown et al., 2016, Staudt and Stainier, 2012, Jia et al., 2023). Next, the cardiac cone elongates and transforms into the linear heart tube by 24 hpf, with the atrial end positioned to the left of the midline (**Figure 7C**). Subsequently, the heart tube continues its leftward migration and initiates looping. By 48 hpf, cardiac looping is complete, and the two chambers of the heart, the ventricle and the atrium, have

formed, consisting of an endocardial and a myocardial tissue layer (Brown et al., 2016, Stainier, 2001).

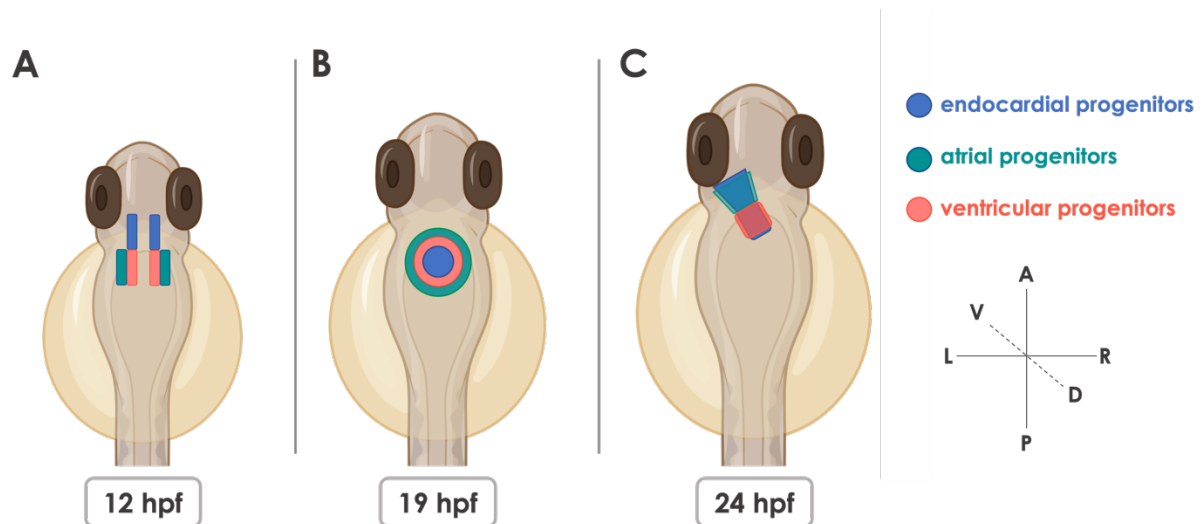


Figure 7 | Zebrafish heart development. Schematic illustrating the sequential developmental steps of the zebrafish heart formation before looping. (A) At 12 hpf, the myocardial progenitors are located in the posterior half of the anterior lateral plate mesoderm (ALPM), with atrial precursors being enriched more laterally compared to ventricular precursors. Endocardial progenitors are found anteriorly in the ALPM, more medially than the myocardial precursors. (B) By 19 hpf, endocardial and myocardial progenitors migrate to the midline, where they subsequently fuse, resulting in the formation of the cardiac cone. (C) By 24 hpf, the cardiac cone telescopes out to create the linear heart tube, with the myocardium surrounding the endocardium. A: anterior, P: posterior, L: left, R: right, V: ventral, D: dorsal, hpf: hours post-fertilisation. (Adapted from (Staudt and Stainier, 2012) and created with Biorender.com).

1.5.2 Atrioventricular valve morphogenesis in zebrafish

The zebrafish atrioventricular (AV) valve separates the ventricle from the atrium, ensuring the unidirectional flow of blood within the heart (O'Donnell and Yutzey, 2020, Henderson et al., 2020). AV valve morphogenesis initiates at 36 hpf with a significant decrease in the cell volume of EdCs specifically located at the area where the future AV valve forms. The process of AVC EdC volume reduction depends on the activity of the transient receptor potential channels Trpp2 and Trpv4, as well as Notch signalling, and occurs in response to mechanical forces (Vignes et al., 2022). The shrinking of AVC EdCs is followed by the formation of an EdC cluster at the level of the AVC within the single-layered endocardial sheet at 48 hpf (**Figure 8**) (Steed et al., 2016a, Steed et al., 2016b, Pestel et al., 2016).

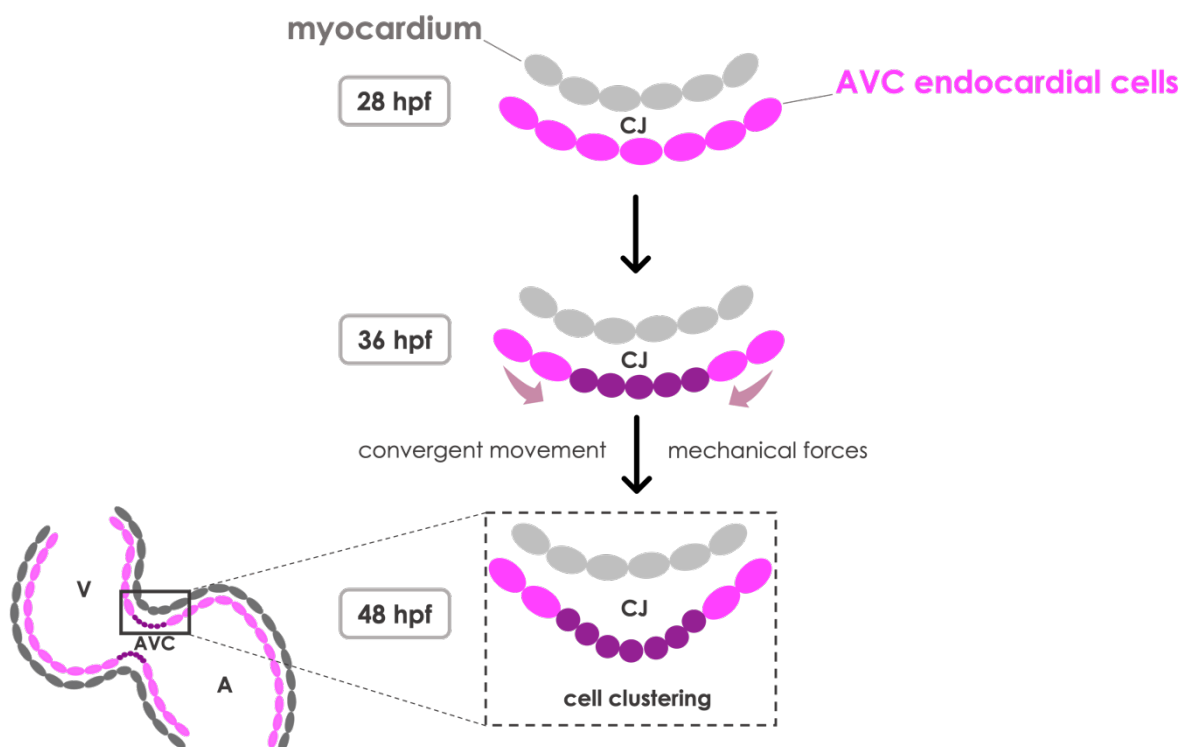


Figure 8 | AVC EdC volume decrease underlines the initiation of zebrafish heart AV valve morphogenesis. Schematic illustrating the event of AVC EdC shrinking after 36 hpf, resulting in EdC clustering. CJ: cardiac jelly, V: ventricle, AVC: atrioventricular canal, A: atrium, hpf: hours post-fertilisation.

Concurrently with these events, a mechanosensitive tissue convergence movement towards the AVC takes place between 36 and 48 hpf (**Figure 9**) (Boselli et al., 2017), indicating that the localised reduction in AVC EdC volume can act as a pulling force on the surrounding cells, promoting tissue convergence and EdC clustering (Vignes et al., 2022).

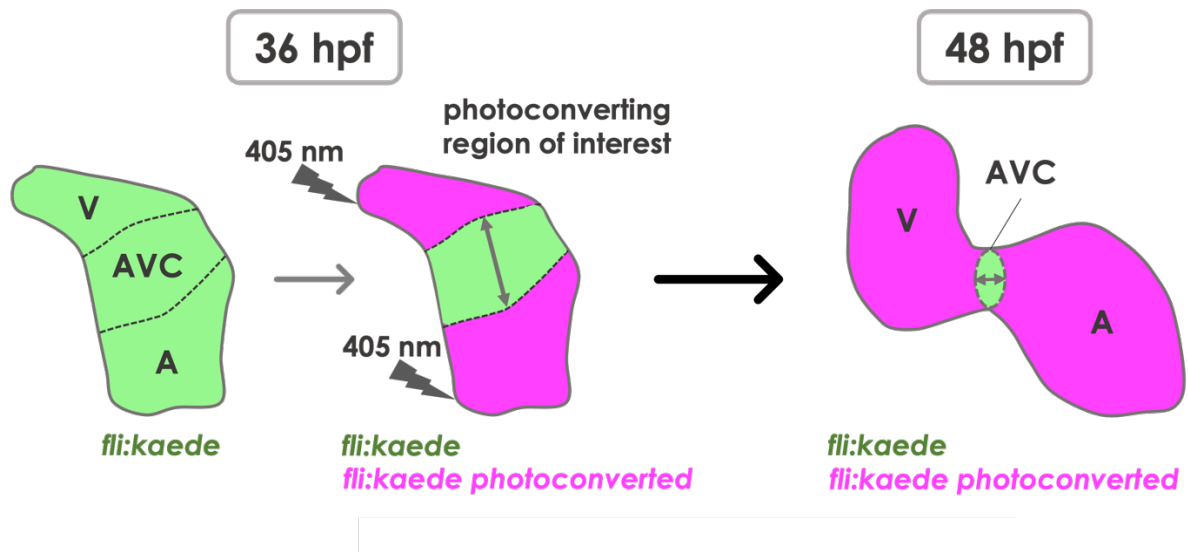


Figure 9 | Tissue convergence towards the AVC occurs between 36 and 48 hpf. The schematic depicts photoconversion experiments, suggesting that EdCs move towards the AVC between 36 and 48 hpf. The two chambers of a *fli:kaede* heart undergo photoconversion (green to red) at 36 hpf using 405 nm UV light. The same heart is imaged at 48 hpf to assess the photoconverted tissue's movement. V: ventricle, AVC: atrioventricular canal, A: atrium, hpf: hours post-fertilisation. (Adapted from (Boselli et al., 2017)).

Clustered EdCs subsequently extend basal protrusions into the cardiac jelly (CJ), which they ultimately invade by 56 hpf. After migrating into the CJ, EdCs undergo a striking transition, losing endothelial characteristics, while showing mesenchymal features. These cells then proliferate to form the endocardial cushions, which undergo extensive remodelling before the valve leaflets emerge (**Figure 10**) (Steed et al., 2016b, Pestel et al., 2016, Gunawan et al., 2019).

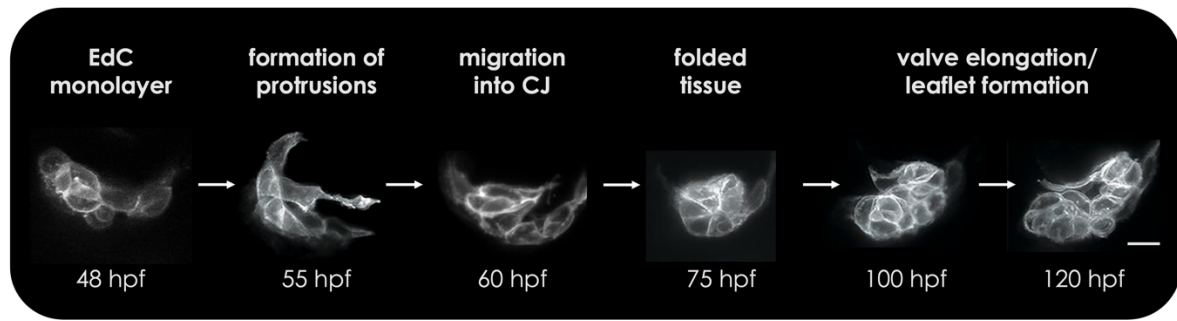


Figure 10 | Formation of the superior AV valve leaflet in zebrafish. Images of EdCs located at the superior AV valve leaflet and marked by *Tg(nfatc1: GAL4);Tg(UAS: EGFP -CAAX)*, at different developmental stages. At 48 hpf, AVC EdCs organise into a monolayer. By 55 hpf, these cells extend protrusions into the CJ, followed by a collective EdC movement into the CJ at 60 hpf. At 75 hpf, the EdCs form a folded structure, ultimately elongating to shape the superior valve leaflet. EdC: endocardial cell, CJ: cardiac jelly, hpf: hours post-fertilisation. Scale bar: 10 μ m. (Adapted from (Gunawan et al., 2019)).

A recent study shows that the leaflet morphology is initially established through delamination, which can occur as early as 72 hpf. After this process, there is ensuing valve elongation, accompanied by the proliferation of abluminal hinge cells located at the base of the valve. The resulting cells ultimately move into the valve interstitial space, becoming valve interstitial cells, commonly referred to as VICs (**Figure 11**) (Chow et al., 2022).

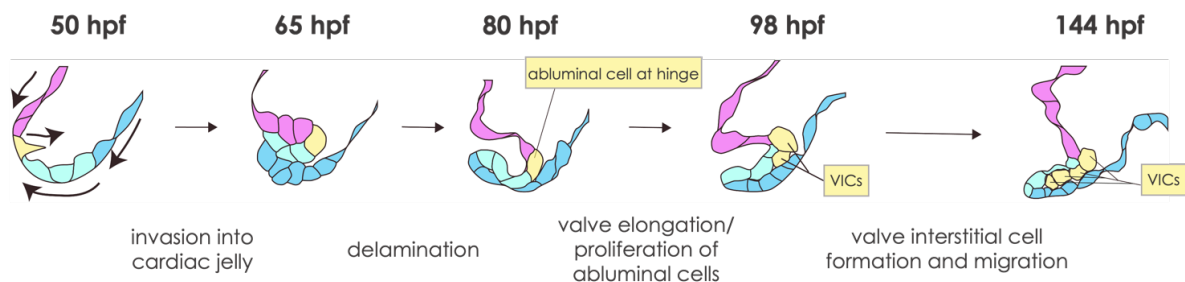


Figure 11 | Delamination serves as the initial mechanism contributing to the establishment of leaflet morphology. Current model for the superior AV valve leaflet development in zebrafish. Ventricular EdCs are represented in magenta, while ventricular EdCs at the upper part of the AVC are in yellow, AVC EdCs in cyan, and atrial EdCs in blue. Arrows at 50 hpf illustrate cell movements. EdCs exhibit basal protrusions into the cardiac jelly (CJ), subsequently invading it after 50 hpf. By 80 hpf, migrating EdCs undergo delamination, followed by valve elongation and the proliferation of abluminal cells. The resulting progeny cells then move into the valve interstitial space, ultimately becoming VICs. VIC: valve interstitial cell, hpf: hours post-fertilisation. (Adapted from (Chow et al., 2022)).

1.6 Mechanical forces underlie heart valve morphogenesis in zebrafish

In the course of embryogenesis, mechanical forces impact the shape, size, position, number, and genetic programmes of cells. Dynamic stimuli are integral to embryonic development, orchestrating tissue patterning, maintaining homeostasis, and determining cell fate. (Heisenberg and Bellaïche, 2013, Mammoto et al., 2013, Hannezo and Heisenberg, 2019).

Mechanical forces continuously provide feedback to the heart valve morphogenetic programmes (Duchemin et al., 2019, Vignes et al., 2022, Steed et al., 2016b, Chow et al., 2022, Fukui et al., 2021), and aberrant blood flow is widely acknowledged as a shared factor in various cardiovascular disorders (Bäck et al., 2013, Hahn and Schwartz, 2009).

In zebrafish, cardiac valve development relies on heart contraction and is governed by mechanical forces generated by the beating heart. Multiple zebrafish mutants have demonstrated the significance of heart-generated mechanical stimuli in valvulogenesis. Remarkably, the complete absence of heart contraction and, consequently, blood flow due to mutations of *cardiac troponin T (tnnt2)* in *silent heart (sih)* mutants results in the absence of tissue convergence and EdC clustering during early morphogenetic stages (**Figure 12**) (Boselli et al., 2017). Therefore, *sih* mutants do not form endocardial cushions. Similarly, *cardiofunk (cfk)* mutants, lacking a functional sarcomeric actin and characterised by absent early blood circulation and cardiac dilation, fail to form endocardial cushions and, hence, functional heart valves (Bartman et al., 2004).

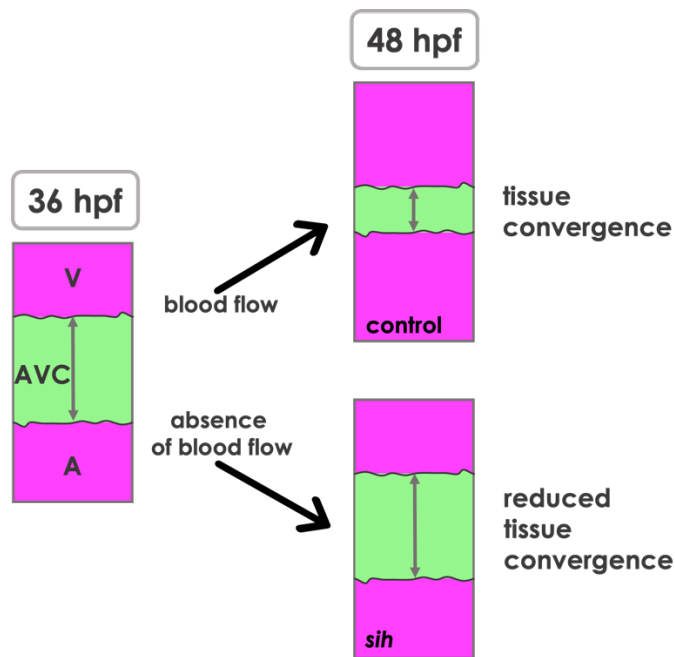


Figure 12 | Tissue convergence towards the AVC is flow- dependent. Schematic illustrating EdCs moving towards the AVC between 36 and 48 hpf through a mechanosensitive process. This mechanism of tissue convergence depends on mechanical forces generated by blood flow; in *silent heart (sih)* mutants, which lack heart contraction, tissue convergence is diminished. *sih*: *silent heart*, V: ventricle, AVC: atrioventricular canal, A: atrium, hpf: hours post-fertilisation. (Adapted from (Boselli et al., 2017)).

In addition, intracardiac haemodynamics and mechanical forces generated by the blood flow emerge as fundamental epigenetic components shaping the development of the zebrafish embryonic heart. Numerous studies suggest that proper cardiac morphogenesis in zebrafish requires distinct flow stimuli, and deviations from these physiological patterns lead to heart valve defects. An effective method to disrupt blood flow and examine potentially abnormal phenotypes involves grafting a glass bead within the zebrafish heart through microsurgery. The effects of bead insertion on blood flow can vary based on the bead's size and placement. This entails the blocking of blood influx into the atrium when the bead is grafted in front of the sinus venosus and the occlusion of blood efflux from the ventricle into the aorta when the bead is inserted at the outflow tract (OFT). These targeted alterations in blood dynamics have been shown to result in dramatic phenotypes, including the absence of heart looping and collapse of the walls of the

inflow and outflow tracts (**Figure 13**), highlighting the important of shear stress in heart morphogenesis (Hove et al., 2003).

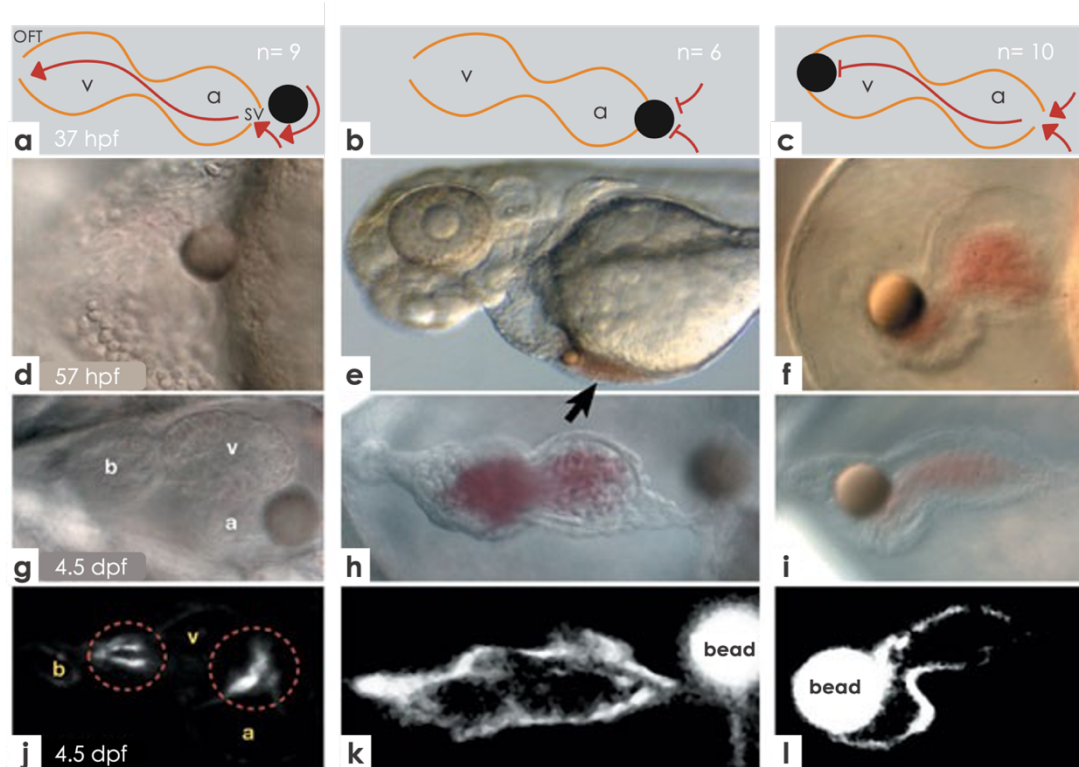


Figure 13 | Deviation from the physiological pattern of flow stimuli leads to heart defects. Images of zebrafish hearts from *Tg(tie2::GFP)* embryos, where a glass bead was grafted into different regions of the heart. (a) At 37 hpf, a glass bead was inserted close the sinus venosus without affecting blood flow, or (b) in front of the sinus venosus, blocking blood influx into the atrium, or (c) in front of the outflow tract (OFT), occluding blood efflux into the aorta. (d-f) Successful implantation of the bead was examined after 20 hours later at 57 hpf. (g) Heart and (j) valve (in red dashed circles) development was unaffected with non-occlusive bead insertion, whereas (h, i) heart development was impaired when the blood flow was blocked, leading to the absence of looping and collapsed chambers. In addition, (k, l) valve morphogenesis was defective. V: ventricle, a: atrium, OFT: outflow tract, SV: sinus venosus, b: bulbus, n: number, hpf: hours post-fertilisation, dpf: days post-fertilisation. (Adapted from (Hove et al., 2003)).

Moreover, mechanical forces generated by blood flow regulate the expression of key genes associated with valve formation. Flow patterns change throughout valvulogenesis, depending on the developmental stage, consequently influencing gene expression patterns. Notably, prior to the formation of functional valves, there is a reversing flow between the atrium and the ventricle, which progressively increases at the AVC from 36 to 58 hpf, as the endocardial ring forms. This oscillatory flow triggers the localised expression of genes essential for AV valve morphogenesis, thereby contributing to the specification of the AVC cell identity of

EdCs. Amongst the mechanosensitive AVC EdC markers are the transcription factor Krüppel-like factor 2a (Klf2a) and a zebrafish Notch homologue, Notch1b (**Figure 14**). It is noteworthy that subtle alterations in haemodynamic patterns can have a profound impact on the specific gene expression patterns, leading to valve malformations (Vermot et al., 2009). The intricate interplay between blood flow patterns and gene expression is fundamental for the proper orchestration of valve development.

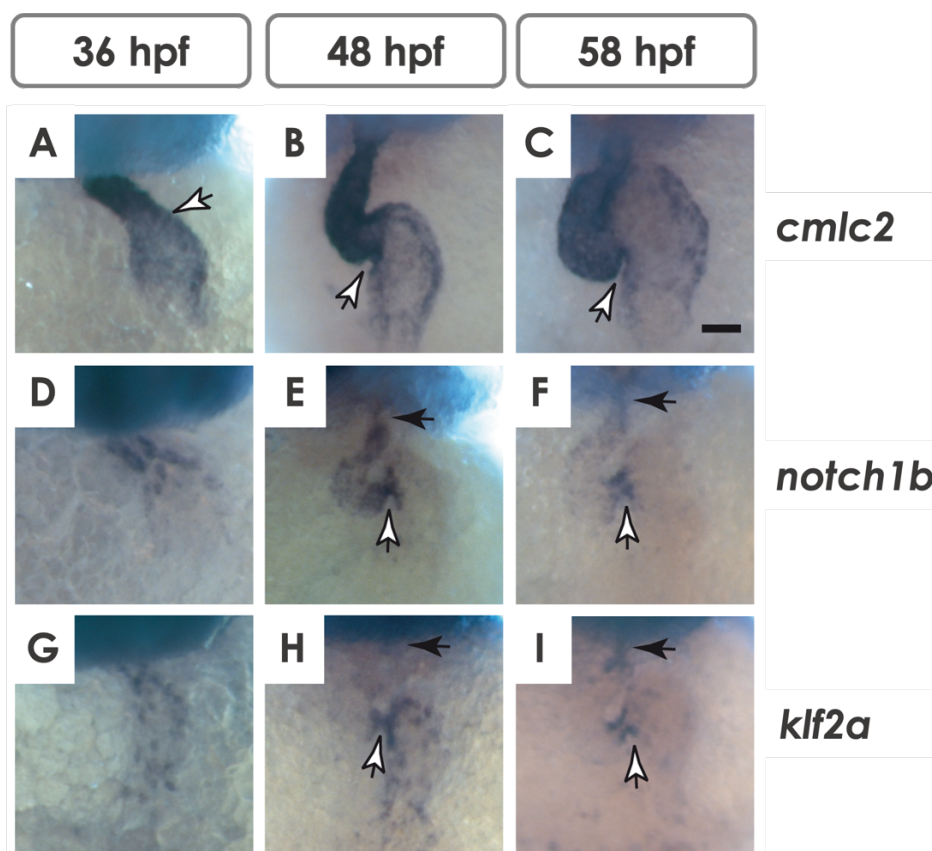


Figure 14 | The expression of key genes becomes localised at the AVC from 36 to 58 hpf, as oscillatory flow increases in amplitude after the EdC ring forms. *In situ* hybridisation images showing the spatial mRNA expression patterns of *cmlc2* (cardiac myosin light chain- 2), *notch1b*, and *klf2a*. (A-C) The expression pattern of *cmlc2* serves as an indicator of the heart's morphology. The mRNA distribution of (D-F) *notch1b* and (G-I) *klf2a* gradually becomes concentrated at the AVC. Black arrows point to the OFT, and white arrows indicate the AVC. Scale bar: 50 μ m. (Adapted from (Vermot et al., 2009)).

1.6.1 Mechanotransduction in heart valve formation

Mechanotransduction is the process through which cells translate mechanical stimuli into biochemical signals, ultimately leading to a cellular response. This mechanism holds a pivotal role in a myriad of physiological procedures throughout all stages of an organism's life, significantly contributing to tissue morphogenesis and the maintenance of cellular homeostasis. Mechanotransduction pathways can be activated by diverse mechanical stimuli, including forces induced by blood flow on the apical cell surface, extracellular matrix and adhesion complex mechanics, as well as the contractility of the actomyosin network, generating forces and stresses (Orr et al., 2006, Martino et al., 2018, Hahn and Schwartz, 2009, Vignes et al., 2022, Fukui et al., 2021, Bailles et al., 2022).

Cells are equipped with molecular structures that enable them to detect changes in mechanical cues. Remarkably, mechanosensitive ion channels are ubiquitous across all kingdoms of life on Earth, spanning eukaryotes, bacteria, and archaea, serving as molecular transmitters of mechanical stimuli. Upon activation, these channels open, initiating osmotic and/or chemical intracellular signals via secondary signalling molecules, such as calcium. Their importance lies in fostering fast signalling events, amongst which are blood pressure and cell volume regulation, touch, and hearing (Gu and Gu, 2014, Jin et al., 2020, Ridone et al., 2019, Martinac, 2012).

In zebrafish heart valve development, mechanotransduction emerges as a fundamental feature for orchestrating cellular and molecular processes. EdCs experience strong physical stimuli due to their direct exposure to blood flow, and osmotic pressure generated by the cardiac jelly (CJ) or extracellular matrix (ECM). Several mechanosensors expressed in the endocardium transduce these signals to regulate morphogenesis. Amongst the essential modulators required for valve development are the mechanosensitive ion channels Piezo1, Piezo2a, as well as

members of the transient receptor potential (TRP) family (Vignes et al., 2022, Chow et al., 2022, Fukui et al., 2021, Juan et al., 2023, Duchemin et al., 2019).

1.6.1.1 Piezo channels

Piezo1 (Fam38A) and Piezo2 (Fam38B) are eukaryotic cation-selective mechanosensitive ion channels (Coste et al., 2010) with a fundamental role in mechanotransduction. Functional homologues have been identified in diverse organisms, spanning invertebrates (Bai et al., 2020), plants (Mousavi et al., 2021, Radin et al., 2021), and protozoa (Coste et al., 2010).

Piezo1 is a large protein, composed of approximately 2520 amino acids, with a molecular weight of 287 kDa. Cryo-Electron microscopy (cryo-EM) studies of mouse Piezo1 have unveiled that it forms a homotrimer, which resembles a three-bladed propeller. Each blade comprises nine repetitive units of four transmembrane helices each. The ion-conducting pore is located at the C-terminus of the protein (Coste et al., 2010, Zhao et al., 2018, Yaganoglu et al., 2023), and demonstrates selectivity for divalent cations, such as calcium (**Figure 15**) (Gottlieb et al., 2012, Coste et al., 2010). Even though Piezo1 and Piezo2 exhibit approximately 42% protein sequence homology, their structures are similar (Wang et al., 2019).

Piezo2 is predominantly found in the peripheral nervous system and is less studied. In contrast, Piezo1 is expressed across various tissues and is primarily present in non-neuronal cell types (Jin et al., 2020). The widespread distribution underscores its importance in mediating mechanotransduction within a broad range of physiological processes (**Figure 16**) (Zhang et al., 2021, Wang et al., 2016, Retaillieu et al., 2015, Ploeg et al., 2021, Liang et al., 2017, Huang et al., 2016, Nguetse et al., 2020, Faucherre et al., 2014, Bae et al., 2013, Velasco-Estevez et al., 2020,

A

Extracellular view

Side view

Intracellular view

B

Mechanotransduction

L15-16

L19-20

THU1

THU2

THU3

THU4

THU5

THU6

THU7

THU8

THU9

Beam

CED

Anchor

OH

Anchor-OH linker

IH-CTD linker

CTD

1361

1690

1300

1342 / 1345

mechanotransduction

Structurally unresolved

100°

140°

100°

90°

Beam (90 Å)

Cap

CED

Blade

Beam

CTD

Anchor

OH

IH

CTD

1361

1690

1300

1342 / 1345

mechanotransduction

Structurally unresolved

100°

140°

100°

90°

Beam (90 Å)

Cap

CED

Blade

Beam

CTD

Anchor

OH

Anchor-OH linker

IH-CTD linker

CTD

1361

1690

1300

1342 / 1345

mechanotransduction

22

Biological processes in which Piezo1 is important	References
Cardiovascular mechanosensation	Duchemin et al., 2019; Juan et al., 2023; Faucherre et al., 2020; Fotiou et al., 2015; Huang et al., 2016; Liang et al., 2017; Lukacs et al., 2015; Ploeg et al., 2021; Retaillieu et al., 2015; Wang et al., 2016; Zhang et al., 2021
Erythrocyte mechanosensation and volume regulation	Albuisson et al., 2013; Andolfo et al., 2013; Bae et al., 2013; Nguetse et al., 2020; Zarychanski et al., 2012; Cahalan et al., 2015; Faucherre et al., 2014
Neural mechanosensation and differentiation	Blumenthal et al., 2014; Pathak et al., 2014; Velasco-Estevez et al., 2020
Epithelial cell mechanosensation	Eisenhoffer et al., 2012; Gudipaty et al., 2017; Kulkarni et al., 2021; Peyronnet et al., 2013
Bladder mechanosensation	Dalghi et al., 2021; Miyamoto et al., 2014
Kidney mechanosensation	Martins et al., 2016
Cartilage mechanosensation	Lee et al., 2014; Lee et al., 2021
Cell adhesion	Agliandolo et al., 2020; McHugh et al., 2010; McHugh et al., 2012
Cell migration	Canales Coutiño and Mayor, 2021; Holt et al., 2021; Hung et al., 2016; Li et al., 2015; Yang et al., 2014
Plant mechanosensation	Radin et al., 2021; Mousavi et al., 2021

Figure 16 | Piezo1 is implicated in a wide range of biological processes. Indicative list of key biological processes in which Piezo1 is important. (Adapted from (Yaganoglu et al., 2023)).

During zebrafish heart morphogenesis, Piezo1 and Piezo2a are critical for the formation of functional AV and OFT valves (Duchemin et al., 2019, Juan et al., 2023, Faucherre et al., 2020). These two mechanosensitive ion channels have been shown to regulate the expression of genes essential for valve development, including *klf2a*, and their absence leads to valve defects (Duchemin et al., 2019, Juan et al., 2023).

1.6.1.2 Transient receptor potential (TRP) channels

Transient receptor potential (TRP) channels are a class of ion channels permeable to various cations, including Ca^{2+} , Mg^{2+} , Na^{+} , K^{+} , and others. They act as sensors for both physical and chemically toxic stimuli and are conserved across yeast, invertebrates, and vertebrates (Nilius and Owsianik, 2011, Zhang et al., 2023, Chen et al., 2020). The mammalian TRP superfamily comprises 28 ion channels, with 27 in humans (Koivisto et al., 2022). These channels are subdivided into seven families, based on their gene sequences, namely TRPC (canonical), TRPP (polycystin), TRPV (vanilloid), TRPM (melastatin), TRPML (mucolipin), TRPA (ankyrin) and TRPN (NOMPC-like) (Nilius and Owsianik, 2011, Zhang et al., 2023).

A typical TRP protein consists of six putative transmembrane segments (S1 to S6), including a pore-forming loop between S5 and S6. The amino and carboxyl termini are localised within the cell cytoplasm, and their length, binding regions and motifs differ significantly amongst the subfamilies (**Figure 17**) (Nilius and Owsianik, 2011).

The majority of TRPs function as non-selective cation channels, with only a few exhibiting high selectivity for Ca^{2+} (Nilius and Owsianik, 2011, Zhang et al., 2023, Samanta et al., 2018). Most TRPs are found at the plasma membrane (Koivisto et al., 2022) and are expressed in a wide range of tissues and cell types, where they engage in a multitude of physiological processes, such as sensory perception and pain sensation (Koivisto et al., 2022, Nilius and Owsianik, 2011), thermoregulation,

bladder function, vision, taste, olfaction, metabolism, immune response, osmosensation, as well as cardiovascular function (Samanta et al., 2018, Song and Yuan, 2010).

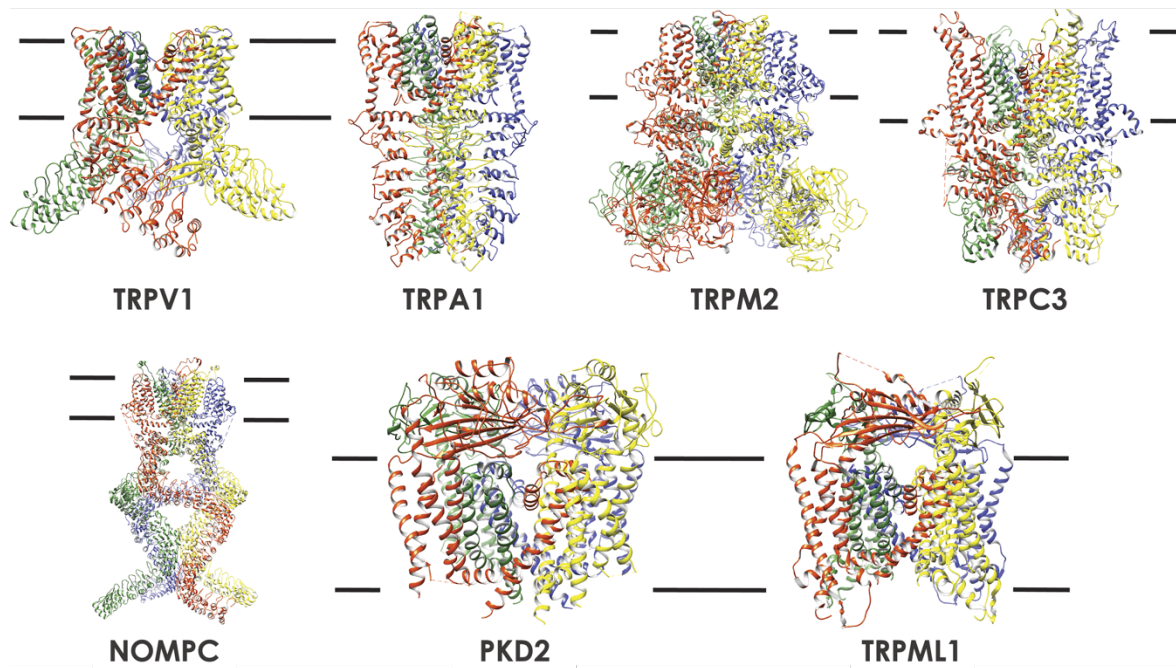


Figure 17 | Representative protein structures of all seven TRP subfamilies. Views of the TRP structures in cartoon mode. The black lines indicate the lipid bilayer of the cell membrane. (Adapted from (Cao, 2020)).

In zebrafish heart development, *Trpp2* and *Trpv4* are expressed in the endocardium, with the distribution of *Trpv4* being enriched in the AVC. These two channels have been associated with the regulation of EdC volume during early heart valve morphogenesis (Vignes et al., 2022), as well as the control of *klf2a* expression, thereby influencing physiological heart valve formation (Heckel et al., 2015). Additionally, several members of the TRP family, namely *Pkd1a*, *Pkd2*, and *Pkd11l1*, are essential to promote AV valve elongation, and their absence leads to AV valve defects (Juan et al., 2023).

1.6.2 Mechanical forces in AVC EdC volume regulation

Mechanical forces and mechanotransduction are indispensable factors driving the morphogenesis of zebrafish heart valves (Chow et al., 2022, Fukui et al., 2021, Vignes et al., 2022, Dye and Lincoln, 2020, Haack and Abdelilah-Seyfried, 2016, Vermot et al., 2009, Steed et al., 2016a, Steed et al., 2016b, Boselli et al., 2017, Heckel et al., 2015, Duchemin et al., 2019, Goddard et al., 2017, Juan et al., 2023). The initial function of mechanical stimuli in valve development is to contribute to the establishment of the AVC.

During early stages, when the zebrafish heart has a tubular structure, EdCs cluster to form the future AV valve-forming region (Steed et al., 2016a, Steed et al., 2016b, Pestel et al., 2016). This clustering event is mediated by a significant localised decrease in EdC volume, which initiates at 36 hpf. The reduction of AVC EdC volume relies on the activity of the Trpp2 and Trpv4 mechanosensitive ion channels, as well as Notch signalling (**Figure 18**), and occurs in response to mechanical forces (Vignes et al., 2022).

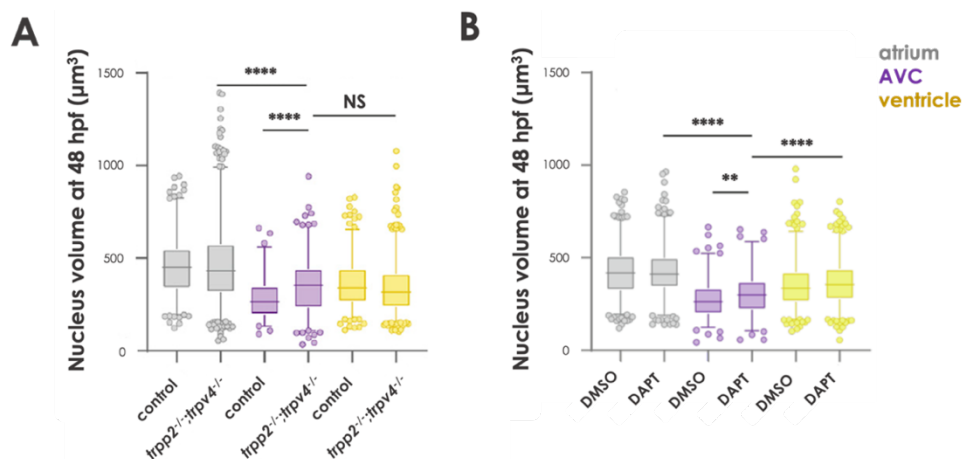


Figure 18 | AVC EdC volume decrease depends on the activity of Trpp2 and Trpv4, as well as Notch signalling. (A) Quantification of EdC volume in controls and *trpp2/trpv4* mutants, in which the AVC EdC volume is significantly increased compared to the controls. (B) Quantification of EdC volume in control DMSO-treated and DAPT-treated embryos, in which the AVC EdC volume is significantly increased compared to the controls. *trpp2*: transient receptor potential polycystin 2, *trpv4*: transient receptor potential cation channel subfamily V member 4, DMSO: dimethyl sulfoxide, DAPT: γ-secretase inhibitor, NS: non-significant, AVC: atrioventricular canal, hpf: hours post-fertilisation. (Adapted from (Vignes et al., 2022)).

Remarkably, AVC EdC shrinking coincides with a global EdC orientation change towards the AVC and correlates with actin filaments (F-actin) remodelling and active cell contractility (**Figure 19**). In addition, cell volume changes are affected by the presence of hyaluronic acid (HA), a negatively charged component of the CJ or ECM, that can generate osmotic pressure and sequester water. Ablation of HA leads to unchanged cell polarity, absence of F-actin remodelling and increased endocardial cell volume at the AVC, suggesting that this glycosaminoglycan is critical for EdC volume regulation and ultimately valve formation (Vignes et al., 2022).

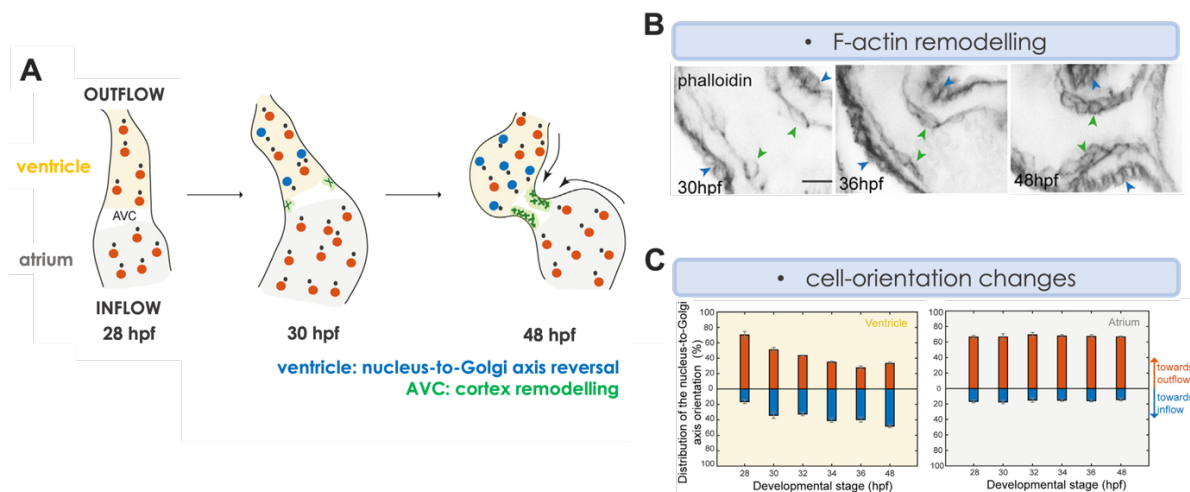


Figure 19 | AVC EdC volume decrease is associated with F-actin remodelling and cell-orientation changes. (A) The reduction in AVC EdC volume during early AV valve morphogenesis coincides with F-actin remodelling and cell-orientation changes specifically in the EdCs located at the AV valve region. (B) Single z-plane immunofluorescence images of phalloidin staining showing increasing signal through time in the AVC (green arrows). Blue arrows indicate the myocardium. Scale bar: 20 μ m. (C) Quantification of cell orientation percentages from 28 to 48 hpf. (Adapted from (Vignes et al., 2022)).

Interestingly, EdCs located at the AVC are equipped with aquaporin-1a.1 (Aqp1a.1) and aquaporin-8a.1 (Aqp8a.1) (Vignes et al., 2022). Aquaporins are membrane-bound passive water channels that enhance membrane permeability (Ibata et al., 2011, Mola et al., 2016). Aqp1a.1 and Aqp8a.1 are enriched in AVC EdCs during early morphogenetic stages and EdC volume decrease (**Figure 20**), and their expression depends on mechanical stimuli generated by blood flow (Vignes et al., 2022).

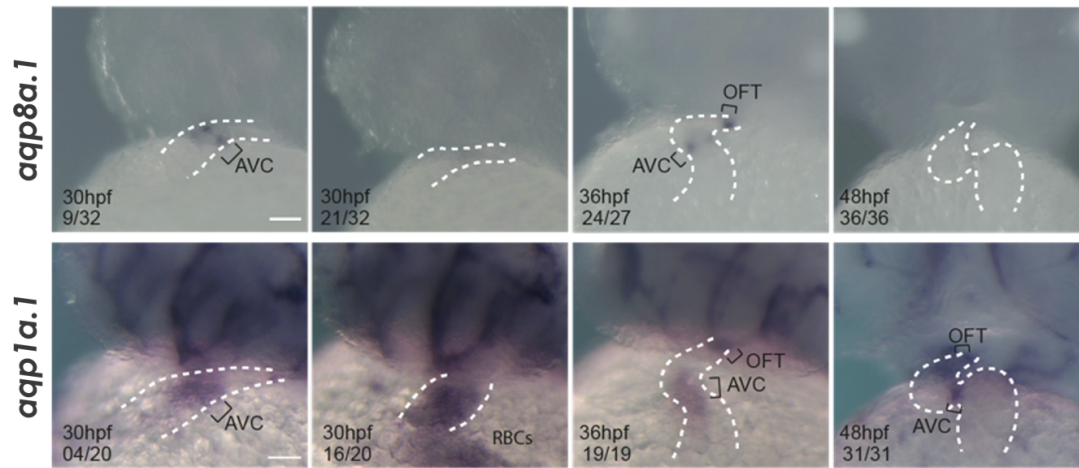


Figure 20 | Expression patterns of *aqp8a.1* and *aqp1a.1* from 36 to 48 hpf. *In situ* hybridisation images showing the spatial mRNA expression profiles of *aqp8a.1* and *aqp1a.1* in the zebrafish heart from 36 to 48 hpf. White dotted lines indicate the heart shape. Scale bar: 50 μ m. (Adapted from (Vignes et al., 2022)).

Overall, the intricate regulation of AVC EdC volume serves as the pivotal initiation phase for the subsequent formation of the AV valve, and it becomes evident that this EdC volume control mechanism is predominantly orchestrated by mechanical forces and mechanotransduction.

1.6.2.1 Aquaporins

Aquaporins are a class of water channel proteins primarily mediating the passive transport of water across the cell membrane in response to osmotic gradients. Despite being commonly known as water channels, they can also facilitate the diffusion of small uncharged solutes, including urea, ammonia, glycerol and hydrogen peroxide, either into or out of cells and organelles (Verkman et al., 2014, Verkerk et al., 2019, Benfenati et al., 2011, Verkman and Mitra, 2000, Calamita, 2023, Markou et al., 2022).

In mammals, 13 family members have been identified, encoded by the genes *AQP0*-*AQP12* (Verkerk et al., 2019, Verkman and Mitra, 2000, Markou et al., 2022, Verkman et al., 2014). These are subdivided into three subfamilies based on their pore

selectivity, namely classical water-selective (AQPs 0, 1, 2, 4, 5, 6, and 8), aquaglyceroporins (AQPs 3, 7, 9, and 10) and unorthodox aquaporins (AQPs 11 and 12) (Verkerk et al., 2019).

Aquaporins are small hydrophobic proteins that organise in membranes as homotetramers. Each monomer, of approximately 30 kDa, functions independently as a water pore (Shi et al., 1994). It comprises six transmembrane α -helices, two half-helices, and a central pore, with the amino and carboxyl termini localised within the cell cytoplasm (**Figure 21**) (Verkerk et al., 2019, Verkman and Mitra, 2000, Markou et al., 2022).

Expressed in various tissues and cell types throughout the organism (Verkerk et al., 2019), aquaporins play a crucial role in diverse physiological processes, spanning cardiac water homeostasis (Verkerk et al., 2019), brain volume homeostasis (Benfenati et al., 2011), glymphatic system function (Mestre et al., 2018), and whole-body water homeostasis via the kidneys (Su et al., 2019).

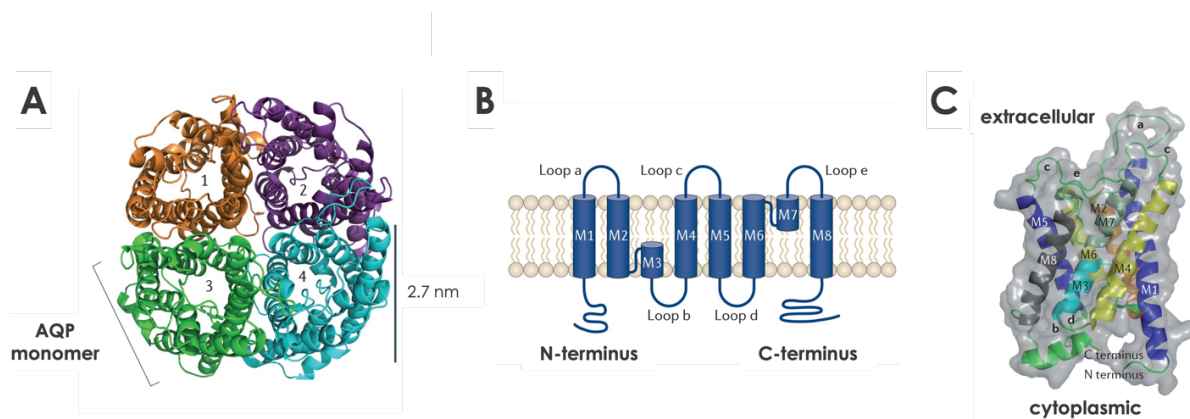


Figure 21 | Aquaporin structure. Aquaporins are small, passive water channels that organise in membranes as homotetramers. (A) Top view of the extracellular part of the bovine aquaporin-1 (AQP1) homotetramer, featuring labelled monomers 1-4. (B) Schematic illustration of AQP monomer membrane topology, and (C) a bovine AQP1 monomer, with six transmembrane α -helices (marked as M1, M2, M4-M7 and M8), two half-helices (labelled M3 and M7) and five connecting loops (indicated as a-e). The N- and C- termini are found in the cytoplasm. (Adapted from (Verkman et al., 2014)).

1.6.2.1a Regulation of aquaporins

Aquaporins can enhance membrane permeability (Ibata et al., 2011, Mola et al., 2016), accelerating regulatory cell volume changes, based on the osmotic and hydrostatic gradient across the cell membrane (Mola et al., 2016). Mammalian aquaporins are regulated through various mechanisms to facilitate dynamic fluid homeostasis. These mechanisms operate at both the gene and protein levels, primarily controlling the abundance of aquaporins on the cell surface and thereby influencing membrane permeability (Markou et al., 2022).

Specifically, the regulation of aquaporin gene expression results in protein level changes, affecting the presence of aquaporins on the cell membrane. This process is inherently slow due to the necessity for gene transcription, protein translation, and the trafficking of the protein from the endoplasmic reticulum membrane, across Golgi, and to the plasma membrane (Markou et al., 2022).

In contrast, a more rapid regulatory mechanism involves the subcellular relocalisation of already existing aquaporins (Markou et al., 2022). Vesicular trafficking is a prevalent biological mechanism that regulates the activity and function of numerous membrane protein families, including subfamilies of aquaporins such as AQP2, AQP4, and AQP6 (Kitchen et al., 2020, van Balkom et al., 2002, Rabaud et al., 2009). This can be facilitated by alterations in the internalisation rates through endocytosis and subsequent return via recycling endosomes. Alternatively, it can occur through the exocytosis of specialised "storage" vesicles (Markou et al., 2022).

Finally, post-translation modifications, including protein phosphorylation or protein-protein interaction, can serve as additional mechanisms for modulating aquaporins and thus membrane permeability (Markou et al., 2022).

Remarkably, calmodulin (CaM) is a ubiquitous regulatory protein reported to control the function of aquaporins and diverse other membrane channels in response to intracellular Ca^{2+} concentration changes (Reichow et al., 2013, Vogel, 1994). CaM can directly bind to various aquaporins, such as AQP0, AQP4 and AQP6 (Reichow et al., 2013, Kitchen et al., 2020, Rabaud et al., 2009, Reichow and Gonen, 2008), which may be located in intracellular vesicles (AQP4 and AQP6) (Kitchen et al., 2020, Rabaud et al., 2009), and regulate their cell-membrane localisation, thus determining their activity and membrane permeability (**Figure 22**) (Kitchen et al., 2020, Conner et al., 2012).

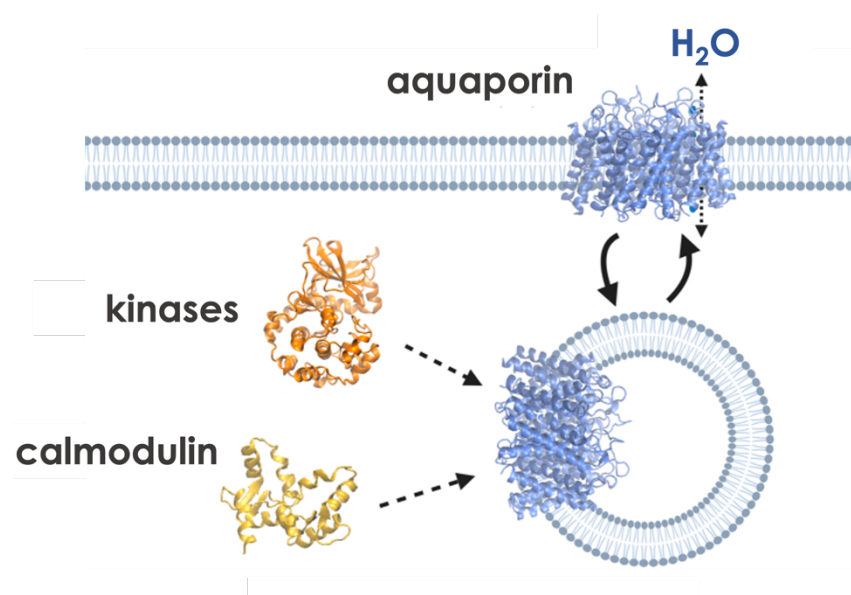


Figure 22 | Aquaporin subcellular relocalisation. Aquaporins can be relocalised between intracellular vesicles and the plasma membrane through direct interaction with calmodulin and/or phosphorylation. (Adapted from (Markou et al., 2022)).

1.6.2.1b Aquaporins in heart function

Aquaporins are implicated in various physiological aspects of heart function, including cardiac water homeostasis (Netti et al., 2016, Verkerk et al., 2019) and energy balance (for aquaglyceroporins) (Skowronski et al., 2007, Palabiyik et al., 2016, Hibuse et al., 2009). Their involvement extends to numerous and pathological conditions, such as heart failure (Xu et al., 1997, Pellicori et al., 2015), pressure-overload hypertrophy (Zheng et al., 1999), myocardial oedema (Li et al., 2015b, Warth et al., 2007), and infective endocarditis (Benoit et al., 2010). The multifaceted contribution of aquaporins underscores their significance in maintaining cardiac health and positions them as potential targets for understanding and addressing diverse cardiovascular disorders.

The presence of aquaporins in the vertebrate heart are contingent upon factors, spanning species, developmental stage, sex, and aging, while different studies might often exhibit discrepancies between species (Butler et al., 2006, Verkerk et al., 2019). In a comprehensive real-time PCR (RT-PCR) assay involving hearts of various species, mRNA expression of AQPs 1, 3, 4, 5, 7, 9, 10, and 11 was detected in human hearts. Rat hearts exhibited mRNA expression for AQPs 1, 4, 6, 7, 9, and 11, while mouse hearts expressed AQPs 1, 4, 6, 7, 8, and 11 (Butler et al., 2006). In the zebrafish heart, Aqp1a.1 and Aqp8a.1 are known to be expressed specifically within the endocardium, during the onset of cardiac valve morphogenesis (Vignes et al., 2022, Koun et al., 2016).

The endocardial expression of Aqp1a.1 and Aqp8a.1, which is notably enriched in the AVC in the early stages of AV valve development (Vignes et al., 2022), suggests that the two water channels may play a pivotal role in regulating the intricate process of AV valve formation, particularly by controlling water flux and EdC volume. However, this aspect has not been investigated.

1.6.2.2 Notch signalling

Notch signalling is a highly conserved cell communication pathway pivotal for determining cell fate and orchestrating tissue growth and patterning. The Notch receptors, along with their ligands, Delta and Serrate (referred to as Jagged in mammals), constitute transmembrane proteins that mediate contact information to adjacent cells. The fundamental principle of Notch signalling relies on proteolytic cleavage, leading to the release of a transcriptionally active intracellular fragment (NICD) (**Figure 23**) (Bray, 2006, Kovall et al., 2017).

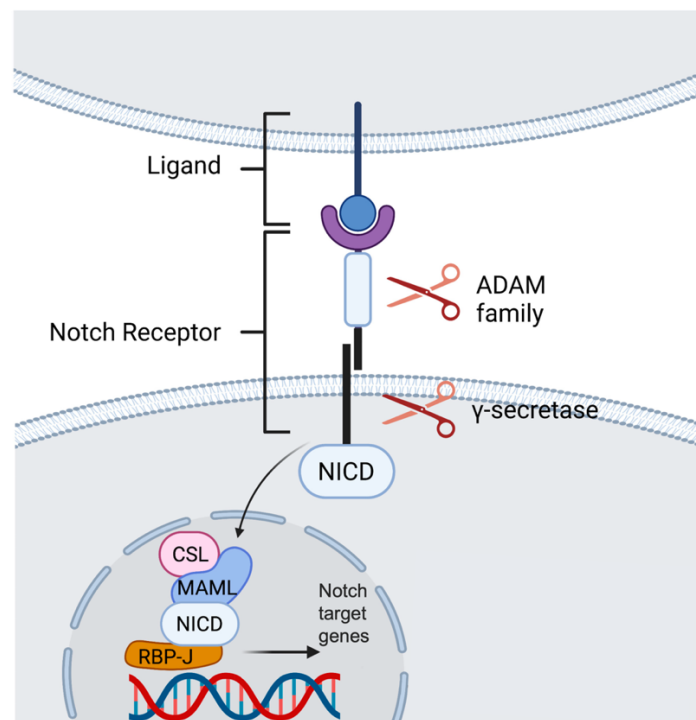


Figure 23 | Notch signalling. The ligand-receptor interaction induces a conformational change in the Notch receptor, triggering a series of proteolytic events. The initial cleavage, mediated by the ADAM family, results in the shedding of the extracellular domain. Subsequently, the second cleavage, facilitated by a γ -secretase complex, occurs within the transmembrane domain. This leads to the release of the intracellular domain (NICD) and its translocation to the nucleus, where it activates the transcription of Notch target genes, along with CSL (CBF1/Suppressor of Hairless/LAG-1; also known as RBP-J) and Mastermind-like protein (MAML). (Adapted from (Andersson and Lendahl, 2014)).

While the conventional view attributes the activation of Notch signalling to the receptor-ligand interaction, mechanical forces can also stimulate the Notch pathway (Caolo et al., 2020, Mack et al., 2017). Multiple studies have demonstrated that

Notch1 is capable of sensing and responding to physical flow forces and shear stress in order to maintain vascular homeostasis (Mack et al., 2017, Jahnsen et al., 2015, Fang et al., 2017). Remarkably, the mechanically activated Piezo1 ion channel has been suggested to trigger Notch1 cleavage in response to calcium influx, subsequently driving the expression of target genes in the hepatic vasculature of mice (Caolo et al., 2020). Moreover, Notch signalling scales with the magnitude of shear stress (Mack et al., 2017), emphasising that mechanical cues play an important role in Notch regulation.

Heart development relies on functional Notch signalling. The complex nature of the heart's structure necessitates the synchronised development of various components, and the Notch cascade is crucial in orchestrating this process (High and Epstein, 2008, Kokubo et al., 2005, Niessen and Karsan, 2008, MacGrogan et al., 2010). Notch signalling contributes to a range of physiological procedures throughout cardiac development, including cell type differentiation, heart region patterning, valve and outflow tract formation, as well as ventricular trabeculation (de la Pompa and Epstein, 2012, Andersson and Lendahl, 2014). Remarkably, mutations in diverse Notch signalling elements have been linked to multiple congenital cardiovascular disorders (Andersson and Lendahl, 2014, MacGrogan et al., 2011, High and Epstein, 2008).

In zebrafish, *notch1b* is prominently expressed and activated in the endocardium during the onset of AV valve morphogenesis (Vermot et al., 2009, Milan et al., 2006). Notably, Notch signalling is essential for regulating of AVC EdC volume and ensuring normal valve development. Disruption of the signalling cascade results in increased AVC EdC volume (Vignes et al., 2022), and impaired valve formation. Conversely, overexpression of activated notch1 leads to hypertrophic endocardial cushions and hypercellular heart valves (Timmerman et al., 2004).

1.6.2.3 F-actin remodelling

Actin filaments (F-actin) and actin crosslinking proteins, along with myosin motors form intracellular networks. By pulling on F-actin, myosin motor proteins generate contractility and active stresses. These subcellular forces are tightly regulated and transmitted to adjacent cells and the extracellular space through adhesion clusters of cadherins or integrins, where F-actin is anchored (**Figure 24**). This control input leads to coordinated cellular deformations, ultimately contributing to tissue shaping and organ formation. Hence, it becomes evident that actomyosin networks orchestrate the intricate process of tissue morphogenesis. (Bailles et al., 2022, Vignes et al., 2022, Collinet and Lecuit, 2021, Munjal and Lecuit, 2014, Lecuit et al., 2011, Munjal et al., 2021).

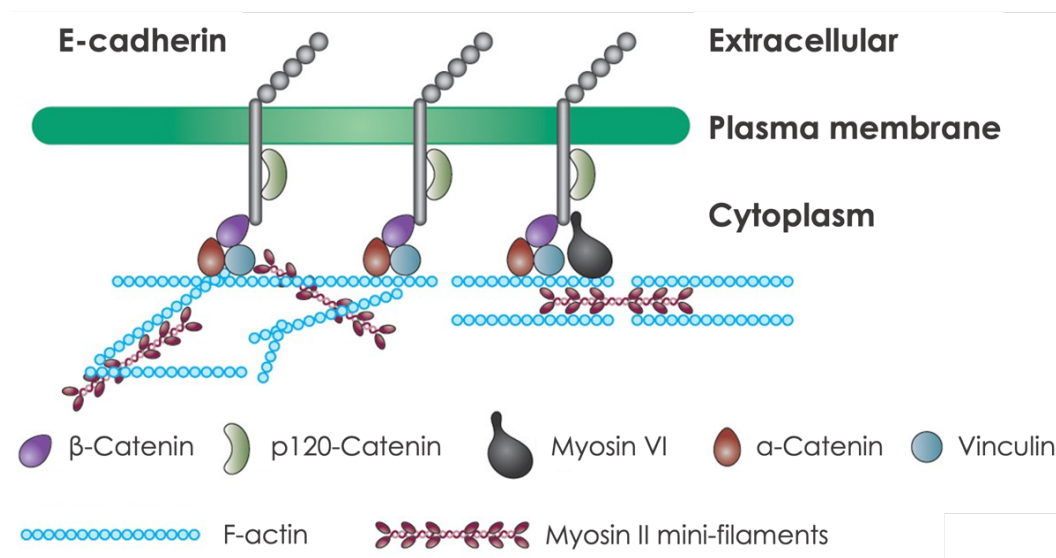


Figure 24 | Schematic of the actomyosin network in epithelial cells. F-actin of the actomyosin network associates with E-cadherin complexes via various mechanisms. These mechanisms include direct binding to α -catenin, interaction with α -catenin associated-proteins such as vinculin, and binding to the motor myosin VI, which can directly attach to E-cadherin. E-cadherin: epithelial cadherin. (Adapted from (Lecuit and Yap, 2015)).

In addition to myosin activation, contractility relies on the geometrical architecture of F-actin, a feature modulated by actin crosslinking proteins. The significance of actin crosslinkers has been emphasised in numerous studies. These proteins connect

filaments, creating networks with diverse structures, geometrical organisations, and contractile characteristics (Laporte et al., 2012, Koenderink and Paluch, 2018, Reymann et al., 2012, Chugh et al., 2017, Pollard and Wu, 2010, Svitkina and Borisy, 1999). Therefore, they prove to be crucial during morphogenesis, contributing to the dynamic arrangement of the actin cytoskeleton and spatiotemporal organisation of actomyosin contraction (Krueger et al., 2019).

In zebrafish, *marcks1a* and *marcks1b* are paralogues of Marcks1, encoding for a crosslinking protein that bundles and stabilises F-actin (Björkblom et al., 2012, Wohnsland et al., 2000). Marcks1 is enriched at the apical membrane of endothelial cells and regulates cortical actin organisation. Its expression has been associated with the regulation of blood vessel diameter and endothelial cell size (Kondrychyn et al., 2020).

In the context of zebrafish heart valve development, AVC EdC volume decrease coincides with F-actin remodelling and active cell contractility, particularly in the cells responsible for forming the AV valve (Vignes et al., 2022). Interestingly, RNA sequencing data from the Vermot lab reveal that *marcks1a* and *marcks1b* are expressed in the embryonic heart during early stages of valvulogenesis (unpublished). These findings indicate that dynamic changes in actin organisation within specific EdCs and potentially involving actin crosslinkers may play a significant role in coordinating the complex process of AV valve formation; however, this aspect has not been further investigated.

1.7 Aims of this thesis

The zebrafish AV valve distinguishes the ventricle from the atrium and establishes the unidirectional flow of blood within the heart. The onset of AV valve morphogenesis at 36 hpf is marked by a significant decrease in the cell volume of EdCs specifically located at the area where the future AV valve forms (Vignes et al., 2022).

During my PhD, I aimed to unravel cellular and molecular mechanisms underlying EdC volume regulation in zebrafish AV valve development. To accomplish this, my research project encompassed three primary Aims:

Aim 1. Investigate the implication of Piezo mechanosensitive ion channels in EdC volume decrease

The reduction of AVC EdC volume is a mechanosensitive process that relies on the activity of the Trpp2 and Trpv4 ion channels (Vignes et al., 2022). However, it remains unknown whether other mechanically activated ion channels are involved.

To address this, I focused on Piezo1 and Piezo2a. In zebrafish cardiac morphogenesis, these channels regulate the expression of genes essential for valve development (Duchemin et al., 2019, Juan et al., 2023) and are critical for the formation of functional AV and OFT valves during late developmental stages, spanning from 72 to 120 hpf (Duchemin et al., 2019, Juan et al., 2023, Faucherre et al., 2020). Nonetheless, their contribution throughout early stages, when the morphogenesis of the AV valve initiates, remains unexplored.

In addition, Piezo1 has been linked to the cell volume homeostasis of red blood cells in zebrafish (Faucherre et al., 2014) and mouse models (Cahalan et al., 2015).

This association indicates a potential central function for Piezo1 in the intricate mechanisms governing AVC EdC volume regulation. Consequently, exploring the role of Piezo1 may provide valuable insights into the regulatory mechanisms underlying AV valve formation and tissue remodelling in zebrafish.

To investigate Piezo1 and Piezo2a's potential effect in early valvulogenesis, I initially examined whether the two channels are expressed in the zebrafish embryonic heart at 36 and 48 hpf by performing *in situ* hybridisation (ISH).

To analyse the impact of Piezo1 on AVC EdC volume decrease, I validated (Yaganoglu et al., 2023) and used a zebrafish Piezo1 overexpression line, where GenEPi, a biosensor for Piezo1 activity, is under the control of the zebrafish heat-shock promoter *hsp70l* (Yaganoglu et al., 2023). This transgenic line allowed me to study the AVC EdC cell volume when Piezo1 is overexpressed and assess whether the expression of genes of interest, including *notch1b*, *aqp1a.1* and *aqp8a.1*, is affected by Piezo1 overexpression.

To further explore the role of Piezo ion channels in the regulation of AVC EdC volume, I used zebrafish mutant lines in which *piezo1* and *piezo2a* carry a deletion in the conserved PFEW domain (Juan et al., 2023), speculated to be involved in channel conductance or gating (Bagriantsev et al., 2014). Using these mutants, I examined the AVC EdC cell volume in the absence of functional Piezo1 and Piezo2a.

Aim 2. Unravel the role of Notch signalling and aquaporins in EdC volume regulation

Notch signalling in EdC volume regulation

Zebrafish heart development relies on functional Notch signalling, and disruption of the Notch pathway results in a defective AV valve (Timmerman et al., 2004). Remarkably, *notch1b* is prominently expressed in the endocardium during the onset of AV valve morphogenesis (Vermot et al., 2009, Milan et al., 2006), and Notch signalling is crucial for modulating AVC EdC volume (Vignes et al., 2022). However, the role of Notch1b and the Notch cascade in this process, along with their interaction with other factors involved in AVC EdC volume decrease have not been studied.

Hence, my goal was to initially uncover the gene-level regulation of *notch1b* and subsequently investigate the impact Notch signalling on the genes and proteins of interest, specifically aquaporins.

To examine the regulation of *notch1b* and identify effectors influencing its expression, I examined its mRNA distribution upon various conditions, including overexpression of Piezo1.

For the analysis of Notch signalling's role in EdC volume control, I disrupted the pathway using N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a well-established γ -secretase inhibitor (Dovey et al., 2001). Following DAPT treatment in zebrafish embryos, I then investigated the expression patterns of *aqp1a.1* and *aqp8a.1*, as well as examined potential changes in the localisation of the water channel proteins.

Aqp1a.1 and Aqp8a.1 in EdC volume regulation

Aqp1a.1 and Aqp8a.1 are expressed in the endocardium during the early stages of AV valve development, and their expression is specifically enriched in the AVC (Vignes et al., 2022). This aspect is very intriguing, especially in the context of cell volume regulation; a possible mechanism for a cell to reduce its volume is by decreasing its water content. Aquaporins can enhance membrane permeability (Ibata et al., 2011, Mola et al., 2016) and accelerate homeostatic volume changes based on the osmotic and hydrostatic gradient across the cell membrane (Mola et al., 2016). Given the negatively charged ECM, which is in direct contact with EdCs and can attract water, the localised mRNA distribution of aquaporins (Vignes et al., 2022) suggests that the two channels may play a pivotal role in regulating AV valve formation by controlling water efflux and promoting EdC volume decrease. Yet, their potential contribution to this event remains unknown.

To investigate the involvement and function of Aqp1a.1 and Aqp8a.1 in EdC volume regulation, I used zebrafish mutant lines, where either both Aqp1a.1 and Aqp8a.1 or one of them is absent. I used these mutant embryos to analyse changes in EdC volume, F-actin distribution, as well as cell polarity. In addition, I examined the morphology of the heart valves to identify potential valve defects.

I then proceeded to explore the localisation and distribution of Aqp1a.1 and Aqp8a.1 in the developing zebrafish heart. To this end, I assessed several antibodies targeting the two water channels and ultimately generated overexpression lines in which both aquaporins are under the control of the *fli1ep* promoter. I used these transgenic lines to examine the localisation of Aqp1a.1 and Aqp8a.1, as well as the mechanisms involved in regulating their localisation.

Regulatory mechanisms of Aqp1a.1 and Aqp8a.1

Moreover, I investigated potential mechanisms of regulation of the two aquaporins at both the gene and protein levels. On the gene level, I examined the expression patterns of *aqp1a.1* and *aqp8a.1* under various conditions, aiming to understand how their expression is modulated and to identify factors involved in the regulatory process. These conditions included the overexpression of Piezo1 and the disruption of Notch signalling.

On the protein level, I examined potential subcellular relocalisation mechanisms. Initially, my focus was on determining whether mechanical forces, and consequently incoming calcium, can influence aquaporin localisation. To investigate this, I performed microsurgery by grafting a bead into beating hearts that overexpressed either Aqp1a.1 or Aqp8a.1 and assessed the ensuing effects. Additionally, using the Calmodulin Target Database, I examined whether the two proteins can directly interact with the calcium-binding protein calmodulin (CaM). Based on the obtained data, I inhibited CaM using a CaM inhibitor (TFP) and conducted site-directed mutagenesis targeting the putative CaM-binding site, with the aim of unravelling the role of CaM in aquaporin subcellular relocalisation.

Aim 3. Examine the role of F-actin remodelling and actin crosslinking proteins in AVC EdC volume reduction and cardiac morphogenesis

Actomyosin networks and, thereby, actin crosslinking proteins play a crucial role in orchestrating the intricate process of tissue morphogenesis (Collinet and Lecuit, 2021, Krueger et al., 2019, Munjal and Lecuit, 2014). In the context of zebrafish cardiac development, the dynamic regulation of AVC EdC volume decrease during early valve formation coincides with F-actin remodelling and active cell contractility, specifically in the cells responsible for forming the AV valve (Vignes et al., 2022).

While the intricacies of these cellular changes suggest a close interplay between actin dynamics and valve development, the necessity of F-actin remodelling and the implication of actin crosslinkers, such as Marcksl1, in EdC volume modulation have not been investigated.

Hence, I aimed to assess the role of Marcksl1 in F-actin remodelling and, thereby, in AVC EdC volume regulation and cardiac valve formation. To achieve this, I used a zebrafish mutant line lacking Marcksl1. Through analysis of mutant embryos, I evaluated the distribution of F-actin in the absence of Marcksl1 and examined its effect on AVC EdC cell volume modulation. Additionally, I studied the morphology of the mutant hearts to identify potential anomalies in the AV valve.

Piezo1 and Piezo2a are involved in AV and OFT formation during late stages and Piezo1 regulates cell volume in various systems

Notch signalling is required for AVC EdC volume decrease

How AVC EdC volume is regulated ?

Actin crosslinkers and F-actin remodelling orchestrate tissue morphogenesis

Aqp8a.1 and Aqp1a.1 are expressed in the endocardium and can regulate cell volume in other systems

Figure 25 | The PhD project rationale. During my PhD, I aimed to unravel cellular and molecular mechanisms underlying EdC volume regulation in zebrafish AV valve development.

Chapter 2. Results

2. 1 Implication of Piezo ion channels in EdC volume decrease

2.1.1 Piezo1 is involved in EdC cell volume regulation

In zebrafish cardiac morphogenesis, Piezo1 and Piezo2a ion channels are crucial for the formation of functional AV and OFT valves during late developmental stages (Duchemin et al., 2019, Juan et al., 2023, Faucherre et al., 2020). In addition, Piezo1 has been linked to the cell volume homeostasis of red blood cells in zebrafish (Faucherre et al., 2014) and mouse models (Cahalan et al., 2015). However, the role of these mechanically activated channels in AV valve formation during early morphogenetic stages and their potential influence on EdC volume changes remains unknown.

To investigate the implication of Piezo1 and Piezo2a in early valvulogenesis, we first examined whether the two channels are expressed in the developing zebrafish heart during relevant stages. To this end, we performed *in situ* hybridisation (ISH) in wild-type embryos at 36 hpf, corresponding to the onset of AVC EdC volume decrease, and at 48 hpf, just prior to the extension of basal protrusions by EdCs into the cardiac jelly. As shown in **Figure 26**, both *piezo1* and *piezo2a* are expressed in the embryonic heart at these time points. These findings align with data obtained from single-cell RNA sequencing conducted by the Stainier lab (data not shown, personal communication), confirming the endocardial expression of *piezo1* and *piezo2a*. Overall, these data suggest the presence of Piezo1 and Piezo2a during early AV valve formation, indicating a potential role of these channels in the process.

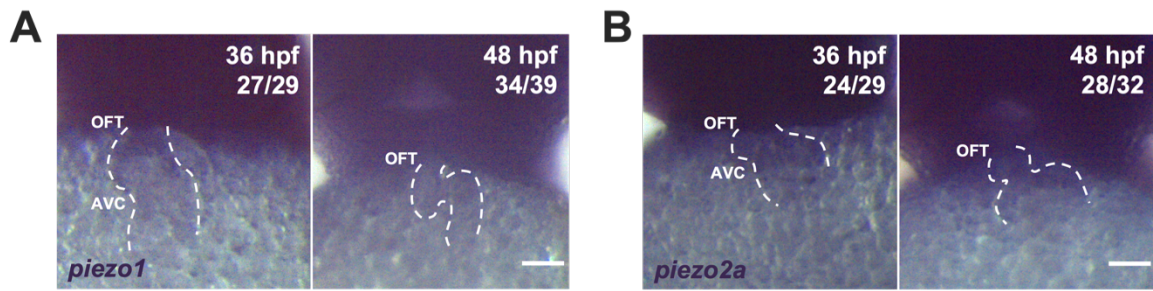


Figure 26 | *Piezo1* and *piezo2a* are expressed in the embryonic heart at 36 and 48 hpf. *In situ* hybridisation images showing the mRNA distribution of (A) *piezo1* and (B) *piezo2a* in the embryonic heart of wild-type embryos at 36 and 48 hpf. White dotted lines indicate the heart shape. Scale bar: 50 µm.

To analyse the impact of Piezo1 on AVC EdC volume decrease, we used a zebrafish Piezo1 overexpression line, in which GenEPi, a biosensor for Piezo1 activity, is under the control of the zebrafish heat-shock promoter *hsp70l*. According to GenEPi's working principle, when mechanical stimuli induce the opening of Piezo1, incoming Ca^{2+} binds to a low affinity GCaMP attached to the C-terminus of the channel, causing an increase in fluorescence (Yaganoglu et al., 2023) (**Figure 27**).

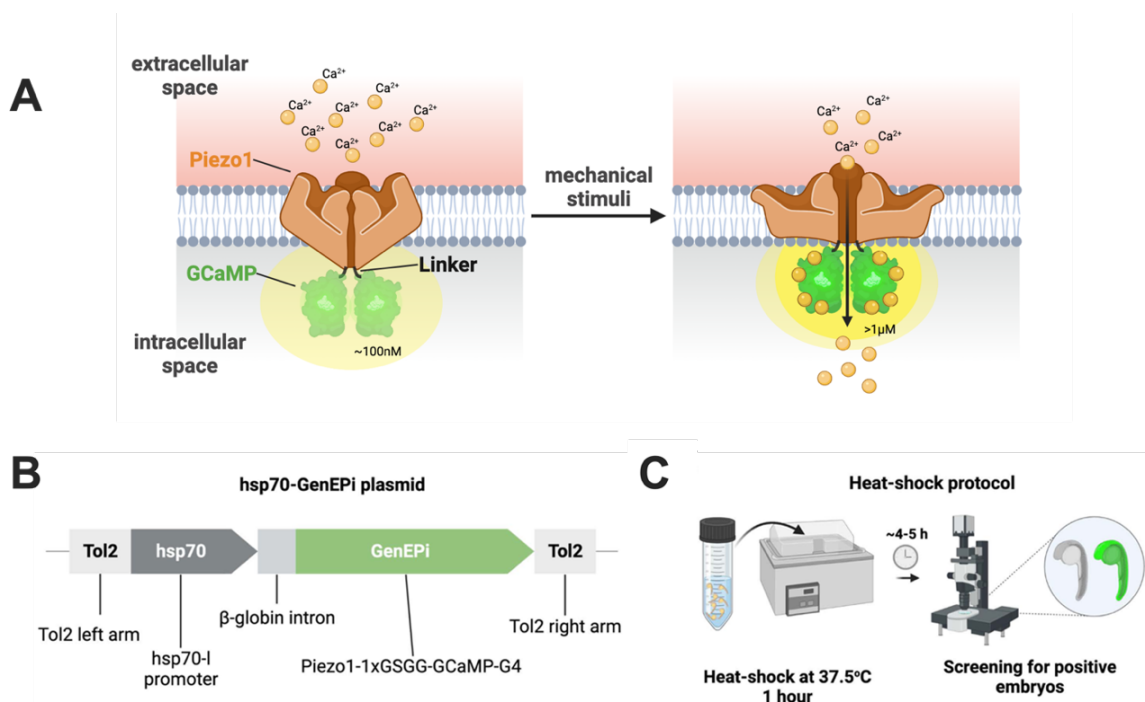


Figure 27 | GenEPi's working principle. (A) Schematic depicting GenEPi's working principle: when mechanical stimuli induce the opening of Piezo1, incoming Ca^{2+} binds to a low affinity GCaMP attached to the C-terminus of the channel, causing an increase in fluorescence. (B) Illustration of the *pTol2-hsp70l:GenEPi* plasmid utilised for zebrafish transgenesis. (C) Schematic outlining the heat-shock protocol used to induce the expression of GenEPi in *Tg(hsp70l:GenEPi)* zebrafish. (Adapted from (Yaganoglu et al., 2023)).

We thus decided to measure the EdC volume in GenEPi-positive fish overexpressing Piezo1. Since the *Tg(hsp70l:GenEPi)* line enables conditional expression of GenEPi and, thereby, functional Piezo1 (Yaganoglu et al., 2023), Piezo1 overexpression was induced following a 1 h heat-shock at 37.5 °C. Control embryos were not subjected to heat-shock. Importantly, there was no variation in the heart rate of individual embryos compared to control non-heat-shocked embryos upon recovery from the heat-shock procedure (Yaganoglu et al., 2023).

To quantify the cell volume of EdCs and overcome the challenges associated with 3D manual segmentation, we developed a robust and quantitative approach based on deep learning. In this method, we used the nucleus volume as a readout of the cell volume. We have previously shown that the nucleus volume directly scales with the cell volume in our system (Vignes et al., 2022). Hence, our focus was on segmenting and quantifying the EdC nuclei volume.

In order to label the nuclei of EdCs and, therefore, facilitate the measurement of the EdC nuclei volume, we conducted immunofluorescence (IF) assay at 48 hpf, using an antibody targeting Fli-1, in deyolked GenEPi-positive and control embryos.

When we measured the EdC volume in GenEPi-positive fish overexpressing Piezo1, we found a significant AVC EdC volume increase compared to control embryos (**Figure 28**). These results suggest that overexpression of Piezo1 affects EdC volume and leads to swelling of AVC EdCs.

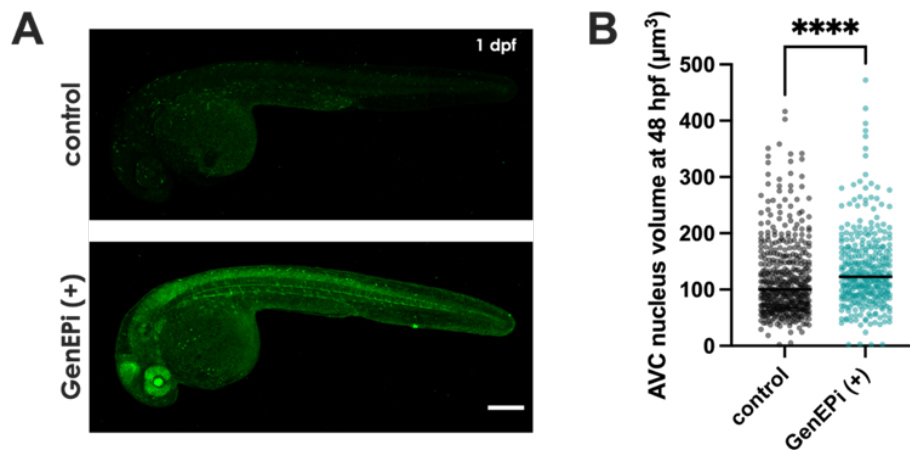


Figure 28 | Piezo1 overexpression affects AVC EdC volume. (A) Representative images of a control (non-heat-shocked) and a heat-shocked GenEPi (+) *Tg(hsp70l:GenEPi)* zebrafish at 1 dpf. Scale bar: 200 μm. (B) Quantification of the nucleus volume in control (N= 13) and GenEPi (+) (N= 12) embryos at 48 hpf. Statistical analysis was performed using a two-tailed unpaired t-test.

To further elucidate the role of Piezo ion channels in the regulation of AVC EdC volume, we used zebrafish mutant lines for *piezo1* (*bns340*) and *piezo2a* (*bns367*) which carry a deletion in the conserved PFEW domain (Juan et al., 2023), speculated to be involved in channel conductance or gating (Bagriantsev et al., 2014). Following the methodology outlined above, we performed IF staining against Fli-1 and subsequently quantified the EdC volume in the absence of functional Piezo1 and Piezo2a at 48 hpf. We observed a reduction in AVC EdC volume in *piezo2a* mutants compared to control embryos, yet it was not statistically significant (**Figure 29**).

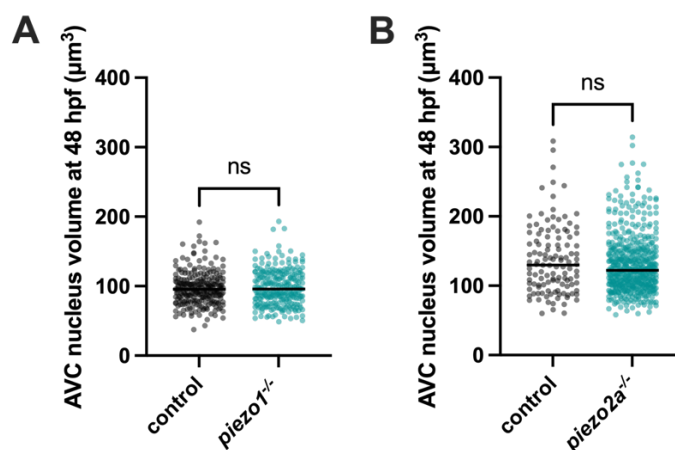


Figure 29 | AVC EdC volume tends to decrease in the absence of Piezo2a. Quantification of the nucleus volume in (A) control (N= 14) and *piezo1* mutant (N= 16) embryos, and in (B) control (N= 10) and *piezo2a* mutant (N= 28) embryos at 48 hpf. Statistical analysis was performed using a two-tailed unpaired t-test.

2.1.2 Piezo1 overexpression downregulates *notch1b* and *aqp8a.1*

Piezo1 and Piezo2a channels control the expression of genes essential for valve formation, specifically *klf2a* and *klf2b* (Duchemin et al., 2019, Juan et al., 2023). Notably, Piezo1 tightly regulates the expression levels of *klf2a* during zebrafish OFT development; in *piezo1* mutants, *klf2a* levels are increased (Duchemin et al., 2019).

Therefore, we aimed to examine whether the expression of genes of interest, including *notch1b*, *aqp1a.1* and *aqp8a.1*, is impacted by Piezo1 overexpression. To achieve this, we conducted ISH in GenEPi-positive fish at 36 and 48 hpf. Our results revealed that the mRNA levels of *notch1b* and *aqp8a.1* are reduced at 36 hpf, coinciding with the most critical time point for the initiation of AVC EdC volume decrease (**Figure 30**). These findings indicate that the overexpression of Piezo1 downregulates the expression of *notch1b* and *aqp8a.1* during a crucial stage for EdC volume regulation.

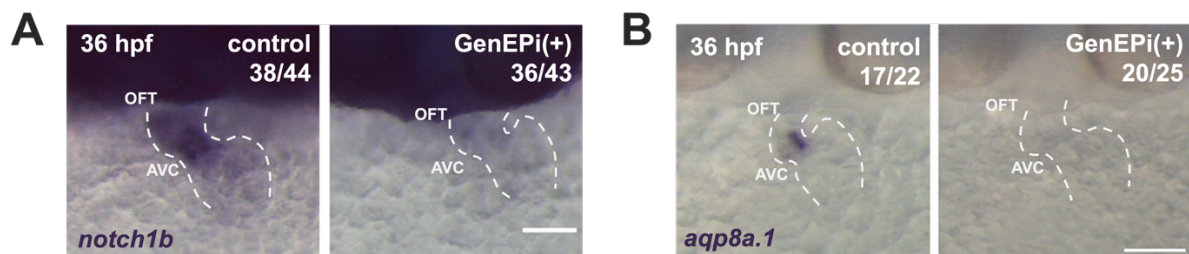


Figure 30 | Piezo1 overexpression leads to downregulation of *notch1b* and *aqp8a.1* at 36 hpf. *In situ* hybridisation images showing the mRNA distribution of (A) *notch1b* and (B) *aqp8a.1* in the embryonic heart of control and GenEPi (+) zebrafish embryos at 36 hpf. White dotted lines indicate the heart shape. Scale bar: 20 μ m.

2.2 Notch signalling in EdC cell volume regulation

2.2.1 Notch signalling controls the expression of *aqp8a.1*

Since overexpression of Piezo1 downregulates the expression of both *notch1b* and *aqp8a.1*, and considering that another aquaporin family member, namely AQP3, has been identified as a transcriptional target of Notch signalling in human keratinocyte differentiation (Guo et al., 2013), our objective was to investigate whether the expression of *aqp8a.1* depends upon the Notch cascade.

To address this, we perturbed the Notch pathway using N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a well-established γ -secretase inhibitor (Dovey et al., 2001), and investigated the mRNA levels of *aqp8a.1* in DAPT-treated embryos. For this purpose, we conducted ISH, which allows for qualitative results, and RNAscope, which allows for quantitative results, at 36 and 48 hpf. Remarkably, we observed a significant downregulation and absence of *aqp8a.1* expression at 36 hpf compared to control DMSO-treated embryos (**Figure 31**). Importantly, the heart rate of individual embryos did not differ amongst DAPT-treated and control embryos (**Figure 32**). These results suggest that the expression of *aqp8a.1* is regulated by Notch signalling and Notch1b acts as an upstream regulator of *aqp8a.1*.

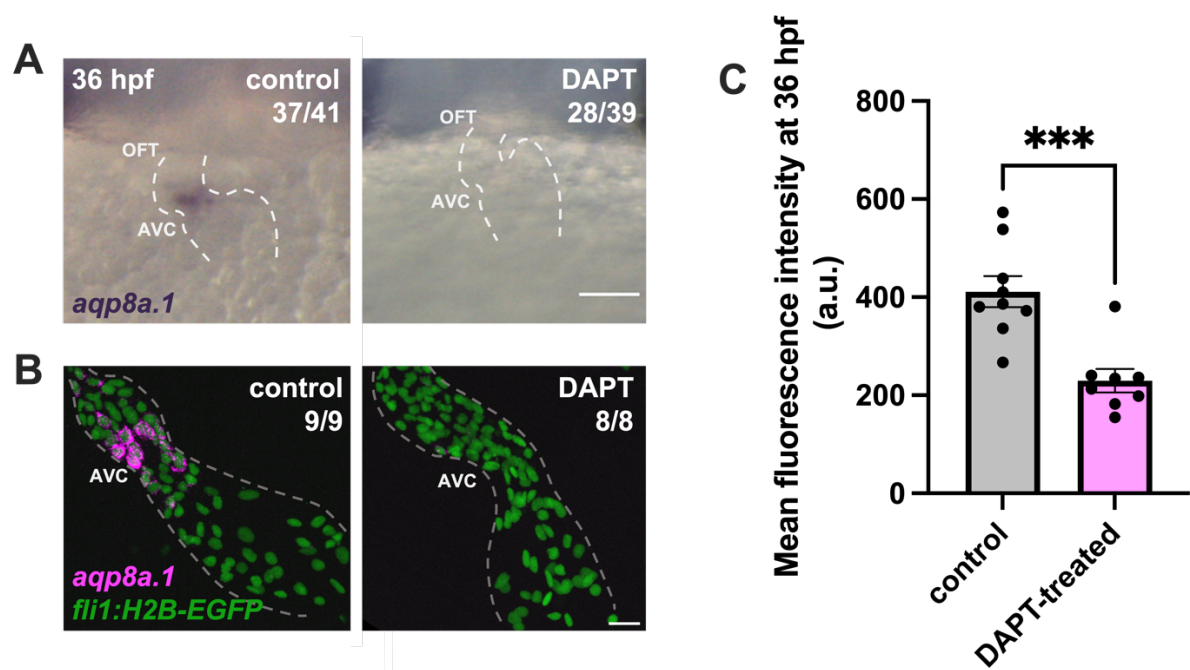


Figure 31 | Disruption of Notch signalling leads to downregulation of *aqp8a.1* at 36 hpf. (A) *In situ* hybridisation and (B) RNAscope images showing the mRNA distribution of *aqp8a.1* in the embryonic heart of control and DAPT-treated embryos at 36 hpf. *Tg(fli1:H2B-EGFP)* zebrafish were used in RNAscope experiments. White/ grey dotted lines indicate the heart shape. Scale bar: 20 μ m. (C) Quantification of the AVC RNAscope fluorescent signal based on the data in (B). a.u.: arbitrary units. Statistical analysis was performed using a two-tailed unpaired t-test.

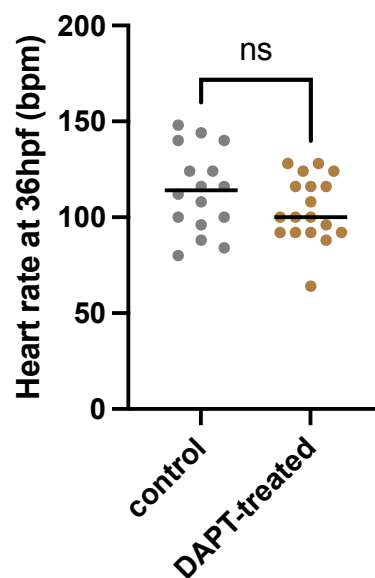


Figure 32 | Heart rate is not affected in DAPT-treated embryos. Quantification of the heart rate in beats per minute (bpm) in control (N= 16) and DAPT-treated (N= 18) embryos at 36 hpf, based on counting the number of beats per minute at room temperature. Statistical analysis was performed using a two-tailed unpaired t-test.

2.2.2 Expression of *notch1b* is independent of Camk2g1 activity

Overexpression of Piezo1 leads to the downregulation of *notch1b*; however, the intermediates of this signalling pathway remain unclear. Recently, the Ca^{2+} /calmodulin-dependent protein kinase Camk2g1 was identified as an intermediate effector of Pkd response upstream of *klf2a* expression, during zebrafish AV valve formation (Juan et al., 2023).

Hence, we decided to examine the potential role of Camk2g1 as an intermediate in the control of *notch1b* expression by Piezo1. For this purpose, we synthesised mRNA encoding a constitutively active (CA) variant of Camk2g1, specifically Camk2g1^{T287D} (Rothschild et al., 2011, Rothschild et al., 2009, Juan et al., 2023), through *in vitro* transcription. Subsequently, we injected this mRNA in wild-type embryos at the 1-cell stage and investigated the expression of *notch1b* at 36 hpf using ISH. As shown in **Figure 33**, we found that *notch1b* expression remained unaffected upon CA Camk2g1 overexpression at 36 hpf, suggesting that its regulation is independent of Camk2g1 activity.

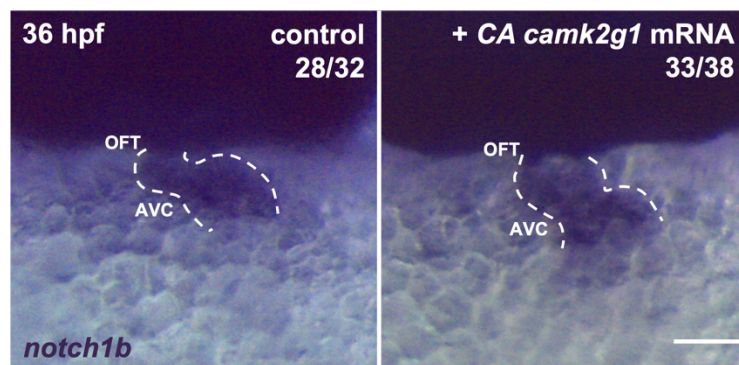


Figure 33 | *Notch1b* expression remains unaffected upon overexpression of CA Camk2g1 at 36 hpf.

In situ hybridisation images showing the mRNA distribution of *notch1b* in the embryonic heart of control and embryos injected with mRNA encoding CA Camk2g1 at 36 hpf. White dotted lines indicate the heart shape. Scale bar: 20 μm .

2.3 Aquaporins in EdC volume regulation

2.3.1 Aqp1a.1 and Aqp8a.1 in EdC volume regulation and AV valve formation

Aqp1a.1 and Aqp8a.1 are present in the endocardium during the onset of AV valve morphogenesis, with their expression being enriched in the AVC (Vignes et al., 2022). This aspect is particularly interesting, especially within the context of cell volume regulation. A potential mechanism for reducing cell volume involves decreasing water content. Aquaporins have the capacity to enhance membrane permeability (Ibata et al., 2011, Mola et al., 2016) and facilitate regulatory volume changes based on the osmotic and hydrostatic gradient across the cell membrane (Mola et al., 2016). Considering the negatively charged ECM, which is in direct contact with EdCs and can attract water, the localised mRNA distribution of aquaporins (Vignes et al., 2022) implies that the two channels may play a central role in regulating AV valve formation by controlling water efflux and promoting EdC volume decrease. Yet, their impact on this process remains unexplored.

To investigate the implication of Aqp1a.1 and Aqp8a.1 in EdC volume regulation, we focused on a zebrafish mutant line where both *aqp1a.1* (*rk28*) and *aqp8a.1* (*rk29*) carry deletions that disrupt the functionality of the encoded water channels (unpublished data from the Phng lab). As the *aqp1a.1;aqp8a.1* mutants expressed an endothelial nuclear marker (*Tg(fli1:EGFP)* background), we arrested mutant and control hearts using the drug 2,3-butanedione monoxime (BDM) and imaged them non-beating at 48 hpf. Remarkably, upon measuring the EdC volume, we found a significant increase in AVC EdC volume compared to control embryos (**Figure 34A**). These data suggest that Aqp1a.1 and Aqp8a.1 are essential for AVC EdC volume reduction.

To further investigate the impact of the absence of Aqp1a.1 and Aqp8a.1 on valve morphogenesis, we examined the heart morphology of *aqp1a.1;aqp8a.1* mutants at 96 hpf, after stopping the heartbeat using BDM. Interestingly, this analysis revealed impaired AV valve elongation (**Figure 34B**), suggesting that Aqp1a.1 and Aqp8a.1 are essential for cardiac valve development.

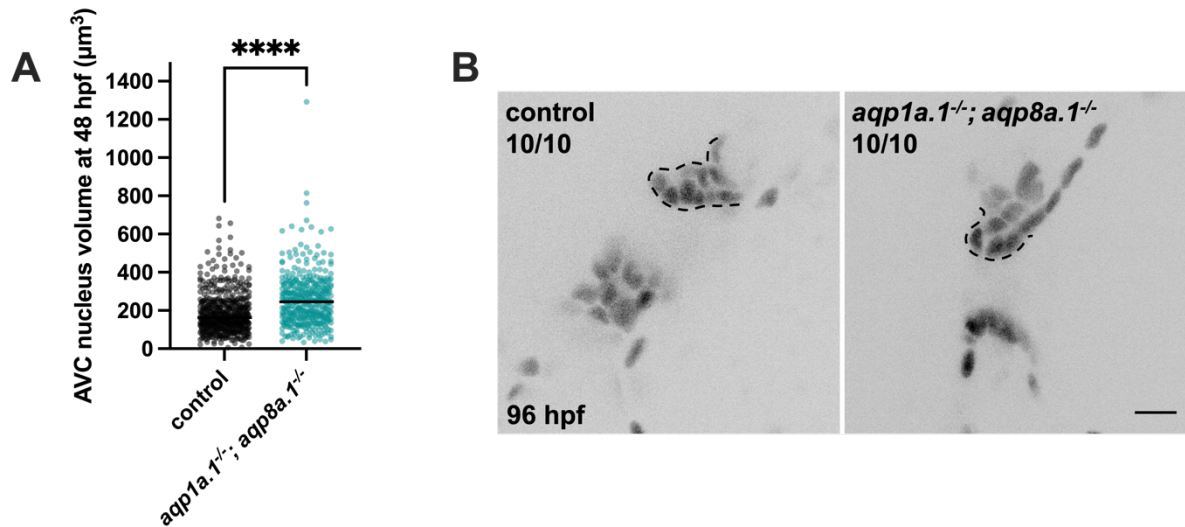


Figure 34 | Absence of Aqp1a.1 and Aqp8a.1 leads to increased AVC EdC volume and valve defects. (A) Quantification of the nucleus volume in control (N= 22) and *aqp1a.1;aqp8a.1* double mutant (N= 16) embryos at 48 hpf. Statistical analysis was performed using a two-tailed unpaired t-test. (B) Single z-plane confocal images of the superior AV valve leaflet of control and *aqp1a.1;aqp8a.1* mutant embryos in *Tg(fli1:EGFP)* background at 96 hpf, where *aqp1a.1;aqp8a.1* mutants show valve elongation defects. Black dotted lines outline the superior AV valve leaflet. Scale bar: 10 μm .

2.3.2 Aqp8a.1 mediates AVC EdC volume and AV valve morphogenesis

Overexpression of Piezo1 leads to downregulation of *aqp8a.1* at 36hpf, when the typical onset of EdC volume reduction initiates, and ultimately results in a significant increase in AVC EdC volume. Therefore, we reasoned that the absence of Aqp8a.1 could be responsible for the observed swelling of AVC EdCs.

To address this, we aimed to determine whether Aqp8a.1 alone can regulate EdC volume changes. We thus focused on *aqp8a.1* mutants and evaluated the EdC volume at 48 hpf. Strikingly, we found a significant increase in AVC EdC volume

compared to control embryos (**Figure 35A**). These data indicate that Aqp8a.1 mediates AVC EdC volume reduction and promotes EdC volume changes.

To examine the role of Aqp8a.1 in valve formation, we investigated the morphology of *aqp8a.1* mutant hearts at 76 hpf. Notably, this analysis revealed AV valve elongation defects (**Figure 35B**), suggesting that Aqp8a.1 is directly involved in valve morphogenesis.

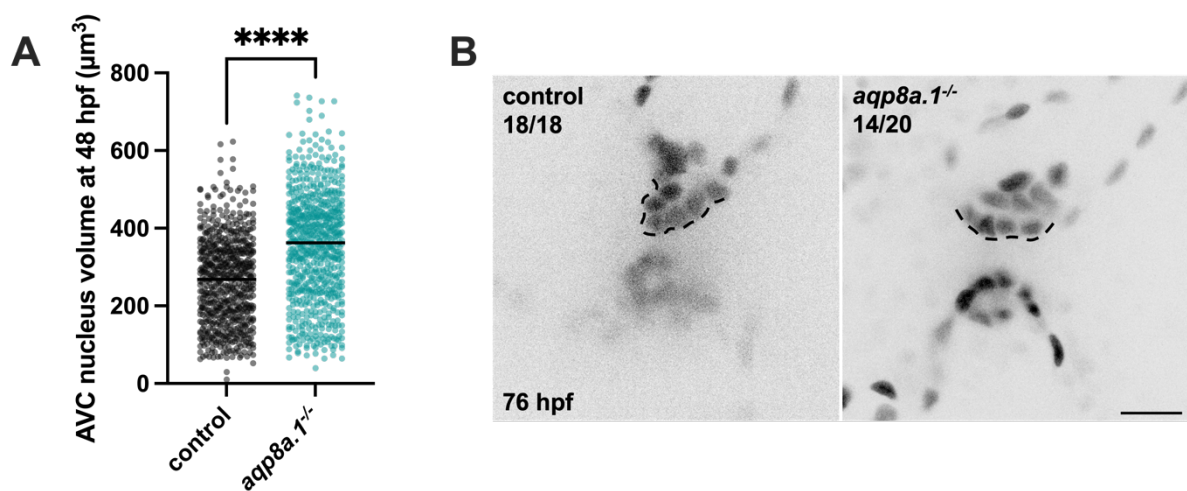


Figure 35 | Absence of Aqp8a.1 alone leads to increased AVC EdC volume and AV valve defects. (A) Quantification of the nucleus volume in control (N= 18) and *aqp8a.1* single mutant (N= 20) embryos at 48 hpf. Statistical analysis was performed using a two-tailed unpaired t-test. (B) Single z-plane confocal images of the superior AV valve leaflet of control and *aqp8a.1* mutant embryos in *Tg(fli1:EGFP)* background at 76 hpf, where *aqp8a.1* mutants show valve elongation defects. Black dotted lines outline the superior AV valve leaflet. Scale bar: 10 μm .

2.3.3 Loss of Aqp8a.1 leads to altered cell polarity

AVC EdC volume decrease coincides with a global endocardial cell orientation shift towards the AVC, highlighting the coordinated EdC movement towards the region where the AV valve forms (Vignes et al., 2022). Therefore, we aimed to examine alterations in cell polarity in *aqp8a.1* mutant embryos, which exhibit increased AVC EdC volume and ensuing AV valve defects.

To visualise axial cell polarity, we used an *aqp8a.1* mutant line expressing markers for the Golgi apparatus and the nucleus (*Tg(fli1:Has.B4GALT1-mCherry)^{bns9}; (kdrl:NLS-EGFP)^{ubs1}* background). Since the use of BDM affects cell orientation (data not shown), we fixed mutant and control embryos at 48 hpf and imaged isolated hearts. We observed altered cell polarity in *aqp8a.1* mutants compared to control embryos. Specifically, in the absence of Aqp8a.1, cell polarity was characterised by a smaller number of ventricular cells having their nucleus-to-Golgi axis pointing towards the inflow, while cell orientation in the atrium remained unchanged (**Figure 36**). These findings indicate a correlation between the absence of Aqp8a.1 and cell orientation, a key feature of AVC EdC volume reduction.

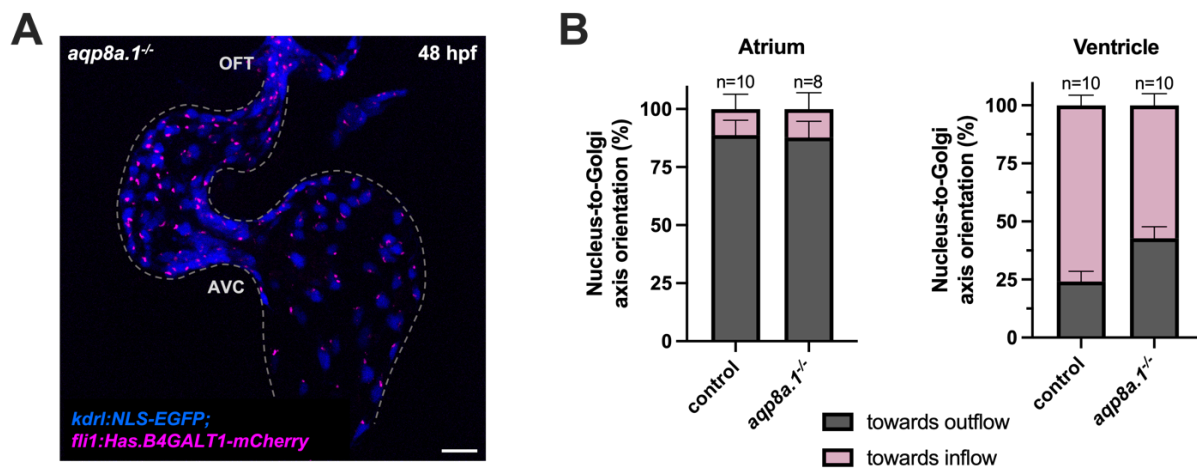


Figure 36 | Absence of Aqp8a.1 leads to altered cell polarity. (A) Representative confocal image of an *aqp8a.1* mutant heart in *Tg(fli1:Has.B4GALT1-mCherry)^{bns9}; (kdrl:NLS-EGFP)^{ubs1}* background at 48 hpf. Grey dotted lines outline the endocardium. Scale bar: 20 μ m. (B) Quantification of cell orientation percentages in control and *aqp8a.1* mutant embryos at 48 hpf. 'n' indicates the number of zebrafish embryos used in each case.

2.4 Regulation of Aqp8a.1 at the protein level

2.4.1 Aqp8a.1 is primarily localised to the cell membrane and the perinuclear region of the EdCs

Considering the pivotal role of Aqp8a.1 in promoting EdC volume decrease and AV valve morphogenesis, our subsequent objective was to investigate the localisation of this water channel within the embryonic zebrafish heart.

To accomplish this, we tested multiple antibodies; however, none of them effectively worked in zebrafish embryos (data not shown). Therefore, we established overexpression lines for Aqp8a.1, namely *Tg(fli1ep:aqp8a.1-mEmerald)* and *Tg(fli1ep:aqp8a.1-mCherry)*. In these transgenic lines, Aqp8a.1 is fused to either mEmerald or mCherry, respectively, under the control of the *fli1ep* promoter. Following the methodology described above (see 2.3.1 *Aqp1a.1 and Aqp8a.1 in EdC volume regulation and AV valve formation*), we arrested hearts overexpressing Aqp8a.1 using BDM and imaged them at 48 hpf. Our analysis revealed that Aqp8a.1 was primarily localised to the plasma membrane and the perinuclear region of EdCs. Additionally, we observed numerous aggregates of the protein, which we hypothesised represent intracellular vesicles (**Figure 37**).

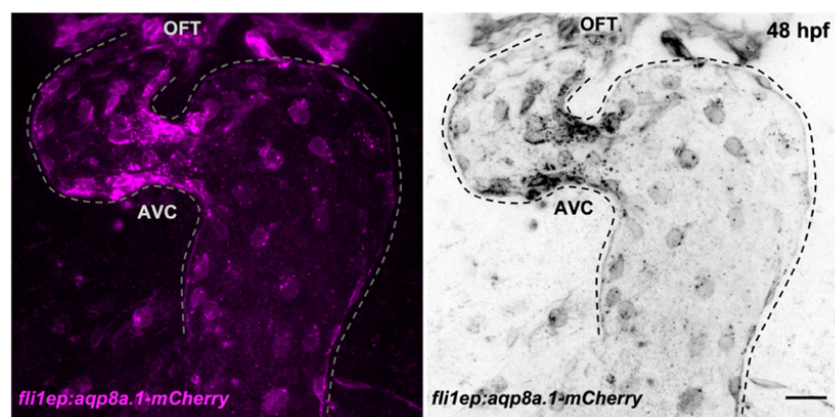


Figure 37 | Aqp8a.1 is primarily localised to the cell membrane and the perinuclear region of the EdCs. Representative confocal image of a *Tg(fli1ep:aqp8a.1-mCherry)* zebrafish heart at 48 hpf (N= 45). Grey/ black dotted lines outline the endocardium. Scale bar: 20 μ m.

To confirm the localisation of Aqp8a.1, we conducted multiple colocalisation assays using known markers of the cell membrane (mCherry-CAAX and vascular endothelial (VE)-cadherin) and the nucleus (nls-mCherry and DAPI). Specifically, we crossed *Tg(fli1ep:aqp8a.1-mEmerald)* zebrafish with either *Tg(kdrl:mCherry-CAAX)* or *Tg(kdrl:nls-mCherry)* transgenics, where mCherry-CAAX and nls-mCherry are under the control of the endothelial-specific promoter *kdrl*. As shown in **Figure 38**, we observed colocalisation between the mEmerald-tagged Aqp8a.1 protein and the mCherry-CAAX signal, indicating the presence of Aqp8a.1 in the plasma membrane of EdCs. Similarly, Aqp8a.1 was found in proximity to the labelled EdC nuclei.

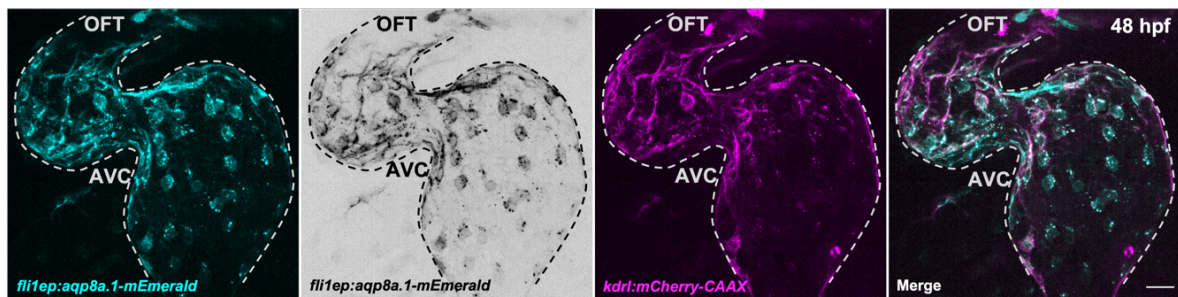


Figure 38 | Aqp8a.1 is localised in the cell membrane. Representative maximum projection of a *Tg(fli1ep:aqp8a.1-mEmerald);Tg(kdrl:mCherry-CAAX)* zebrafish heart at 48 hpf, where colocalisation between the mEmerald-tagged Aqp8a.1 protein and the mCherry-CAAX signal can be observed (N= 8). White/ black dotted lines outline the endocardium. Scale bar: 20 μ m.

To complement these assays, we performed IF targeting VE-cadherin, located at junctions between endothelial cells (Vestweber, 2008), and used the blue-fluorescent DNA stain 4',6-diamidino-2-phenylindole (DAPI) (Kapuscinski, 1995) in *Tg(fli1ep:aqp8a.1-mCherry)* zebrafish at 48 hpf. These stainings validated our previous observations, with partial colocalisation between Aqp8a.1 and VE-cadherin, confirming the membrane localisation of Aqp8a.1, alongside its localisation around the DAPI-labelled EdC nuclei (**Figure 39**).

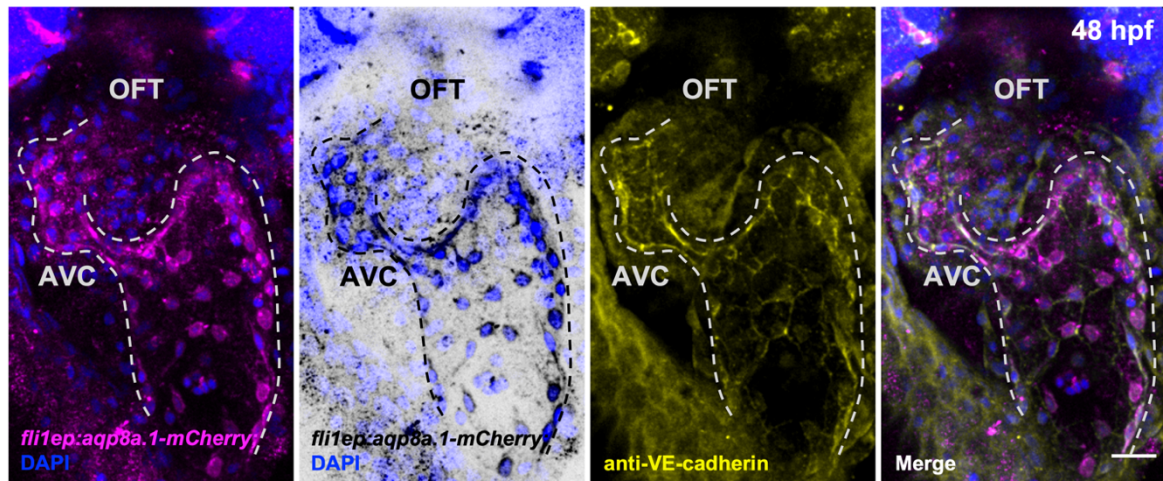


Figure 39 | Aqp8a.1 is localised to the cell membrane and the perinuclear region of the EdCs. Representative partial maximum projection of a *Tg(fli1ep:aqp8a.1-mCherry)* zebrafish heart immunostained for VE-cadherin at 48 hpf (N= 5). Partial colocalisation between the mCherry-tagged Aqp8a.1 protein and VE-cadherin can be observed, alongside its localisation around the DAPI-labelled EdC nuclei. White/ black dotted lines outline the endocardium. Scale bar: 20 μ m.

2.4.2 Aqp8a.1 localisation is controlled by mechanical forces

Intriguingly, despite Aqp8a.1 being expressed under the *fli1* promoter, which would typically result in an even distribution of the protein within EdCs, we observed its evident enrichment at the AVC (**Figure 37**).

At this point, it becomes imperative to consider that mechanical forces and calcium levels are notably elevated at the AVC (Fukui et al., 2021, Juan et al., 2023). This suggests that the localisation of the Aqp8a.1 may be influenced by shear stress and calcium influx. More precisely, we hypothesised that shear stress and incoming calcium stabilise Aqp8a.1 by enhancing its incorporation into the plasma membrane.

To examine whether mechanical stimuli and increased intracellular calcium levels can induce changes in Aqp8a.1 localisation, we employed a bead setup. This method involves the precise surgical insertion of a bead, approximately 30-40 μ m in diameter, into the atrium of the beating heart of anaesthetised zebrafish subjects

(**Figure 40**). The grafting of the bead amplifies the mechanical forces experienced by EdCs, thereby directly triggering calcium influx (Fukui et al., 2021).

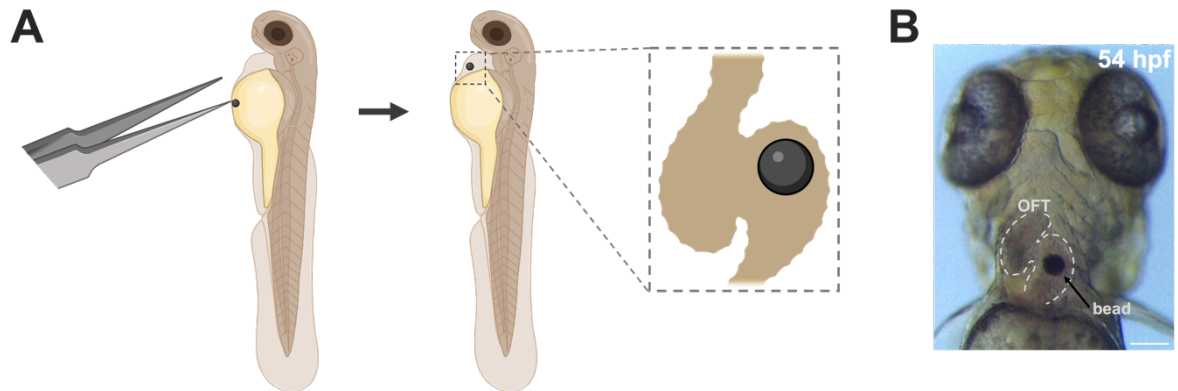


Figure 40 | The bead insertion procedure in zebrafish. (A) Schematic illustrating the sequential steps for grafting a bead into the zebrafish beating heart. (Created with Biorender.com). (B) Brightfield image showing a zebrafish at 54 hpf with a bead successfully inserted into the atrium of its heart. White dotted lines indicate the heart shape. Scale bar: 20 μ m.

To monitor potential changes in the localisation of Aqp8a.1, we initially conducted live imaging of BDM-arrested hearts (see 2.3.1 *Aqp1a.1 and Aqp8a.1 in EdC volume regulation and AV valve formation*) that were overexpressing Aqp8a.1 (using the transgenic line *Tg(fli1ep:aqp8a.1-mCherry)*) at 48 hpf. Subsequently, at 54 hpf, when the zebrafish subjects had fully recovered from the BDM treatment and their heartbeats had resumed, we proceeded to insert a bead into the atrium. Ultimately, at 72 hpf, we imaged the same fish with the heartbeat stopped, aiming to investigate any alterations in Aqp8a.1 localisation. As shown in **Figure 41**, in the presence of the bead, we observed evident changes in the protein's localisation throughout the developing heart, particularly at the AVC and the ventricle. These results indicate that the localisation of Aqp8a.1 is governed by both mechanical forces and intracellular calcium levels.

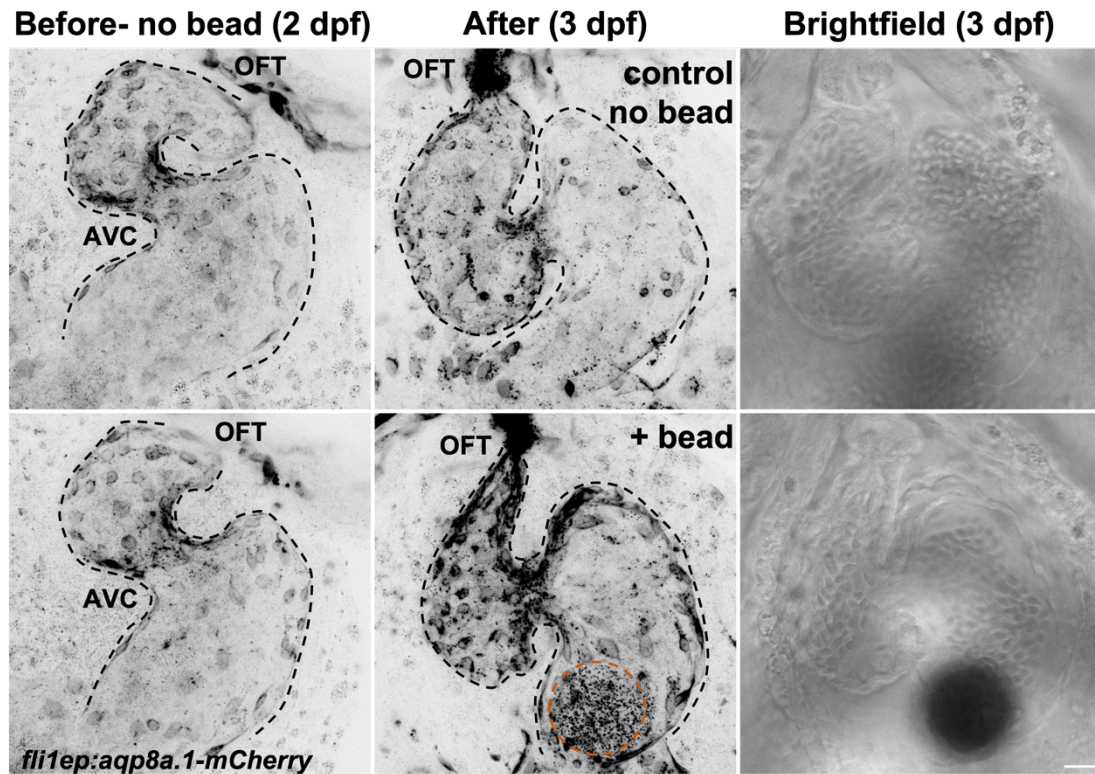


Figure 41 | Aqp8a.1 localisation is controlled by mechanical forces. Confocal images of hearts of *Tg(fli1ep:aqp8a.1-mCherry)* zebrafish at 2 dpf, prior to bead grafting, and at 3 dpf without (N= 5) (upper panel) or with (N= 4) (lower panel) a grafted bead in the atrium, showing evident changes in Aqp8a.1 localisation in the presence of the bead. Black dotted lines outline the endocardium. Orange dotted lines indicate the bead. Scale bar: 20 μ m.

2.4.3 Aqp8a.1 localisation is regulated by calmodulin (CaM) activity

Following these findings, we aimed to further understand the mechanism underlying the localisation changes of Aqp8a.1. To this end, we focused on the Ca^{2+} -binding protein Calmodulin (CaM). CaM is a ubiquitous regulatory protein reported to control the localisation and, thus, the function of aquaporins and diverse other membrane channels in response to intracellular Ca^{2+} concentration changes (Reichow et al., 2013, Vogel, 1994). CaM can directly bind to various aquaporins (Reichow et al., 2013, Kitchen et al., 2020, Rabaud et al., 2009, Reichow and Gonen, 2008), which may be located in intracellular vesicles (Kitchen et al., 2020, Rabaud et al., 2009), and regulate their cell-membrane localisation through vesicular

trafficking, a common mechanism determining the physiological role of many membrane protein families (Kitchen et al., 2020).

To investigate whether Aqp8a.1 can directly interact with CaM, we used the Calmodulin Target Database (Yap et al., 2000). Our analysis revealed a putative CaM-binding site at the carboxyl terminus of the protein, spanning the last residues 235 to 260 (**Figure 42A**). Interestingly, within this site, we identified a potential CaM-binding motif characterised by three leucine residues forming a hydrophobic surface, with charged residues on the other side (**Figure 42B**). This configuration is consistent with a typical characteristic of CaM-binding helices (Tidow and Nissen, 2013).

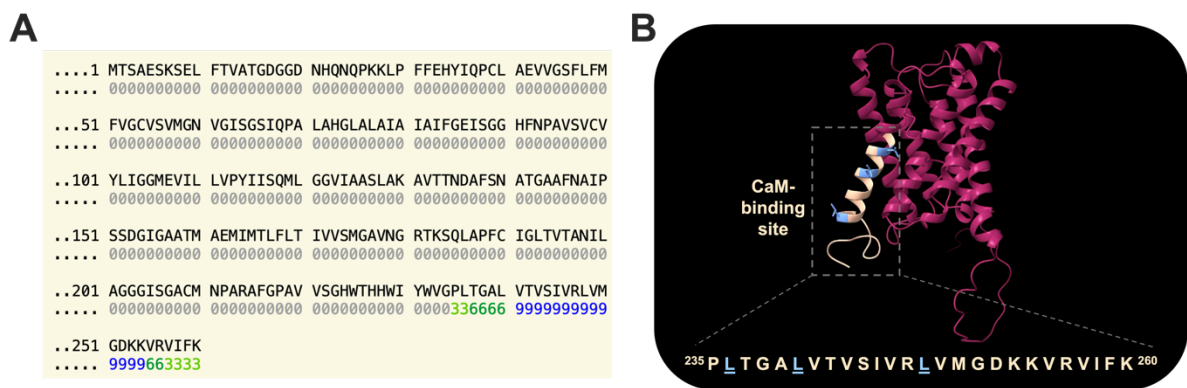


Figure 42 | Aqp8a.1 is predicted to interact with calmodulin (CaM). (A) Aqp8a.1 is predicted to harbour a CaM-binding site at its carboxyl terminus, as indicated by the Calmodulin Target Database (Yap et al., 2000). The highest score, 9, signifies a robust likelihood of interaction between the protein sequence and CaM. (B) Structural schematic of Aqp8a.1, predicted using AlphaFold (Jumper et al., 2021), highlighting the CaM-binding site at its carboxyl terminus. The binding site comprises a potential CaM-binding motif (Tidow and Nissen, 2013) with three leucine residues forming a hydrophobic surface, with charged residues on the other side.

To investigate whether CaM is involved in the localisation changes observed in the presence of the bead (see 2.4.2 *Aqp8a.1 localisation is controlled by mechanical forces*), in response to elevated intracellular calcium levels, we employed the bead insertion approach while inhibiting CaM. Specifically, we followed the bead monitoring methodology outlined earlier (refer to 2.4.2 *Aqp8a.1 localisation is controlled by mechanical forces*). However, in this instance, in addition to grafting

the bead at 54 hpf, we treated the zebrafish subjects with the drug trifluoperazine (TFP), an FDA-approved CaM inhibitor (Kitchen et al., 2020, Taylor et al., 2020), until 72 hpf. Remarkably, we found that inhibition of CaM not only blocks the expected Aqp8a.1 localisation changes induced by the grafted bead but also prevents the enrichment of the protein at the AVC (**Figure 44**). These data suggest that Aqp8a.1 localisation is regulated by CaM activity.

To complement this analysis, we leveraged two key facts: first, that Aqp1a.1 is uniformly distributed throughout the endocardium, without the formation of protein aggregates (**Figure 43A**), and second, that the protein lacks a predicted CaM-binding domain, as indicated by the Calmodulin Target Database (Yap et al., 2000) (**Figure 43B**).

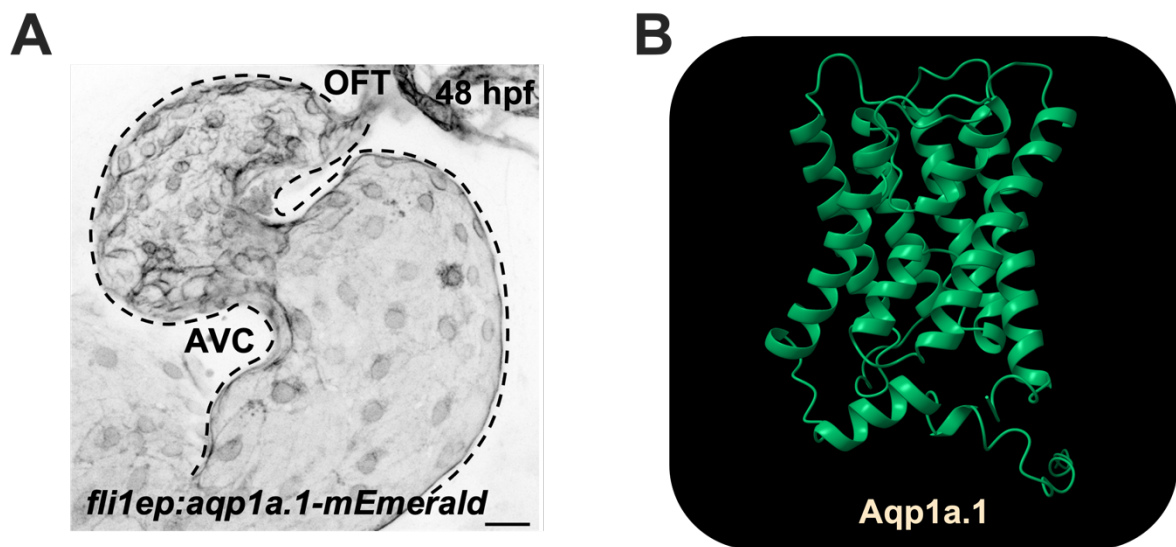


Figure 43 | Aqp1a.1 is not enriched at the AVC and is not predicted to interact with CaM. (A) Representative confocal image of a *Tg(fli1ep:aqp1a.1-mEmerald)* zebrafish heart at 48 hpf, showing the even localisation of the protein in the embryonic heart, without the formation of protein aggregates (N= 22). Black dotted lines outline the endocardium. Scale bar: 20 μ m. (B) Structural schematic of Aqp1a.1, predicted using AlphaFold (Jumper et al., 2021), showing no putative CaM-binding site.

In our experimental approach, we surgically implanted a bead in the hearts of *Tg(fli1ep:aqp1a.1-mEmerald)* zebrafish (as described in 2.4.2 *Aqp8a.1 localisation is controlled by mechanical forces*), which overexpress Aqp1a.1 under the *fli1*

promoter. Significantly, we observed no localisation changes compared to control embryos where no bead was inserted (**Figure 44**). These findings provide additional support for the hypothesis that the presence of the CaM-binding site in Aqp8a.1 is pivotal for the protein's localisation dynamics.

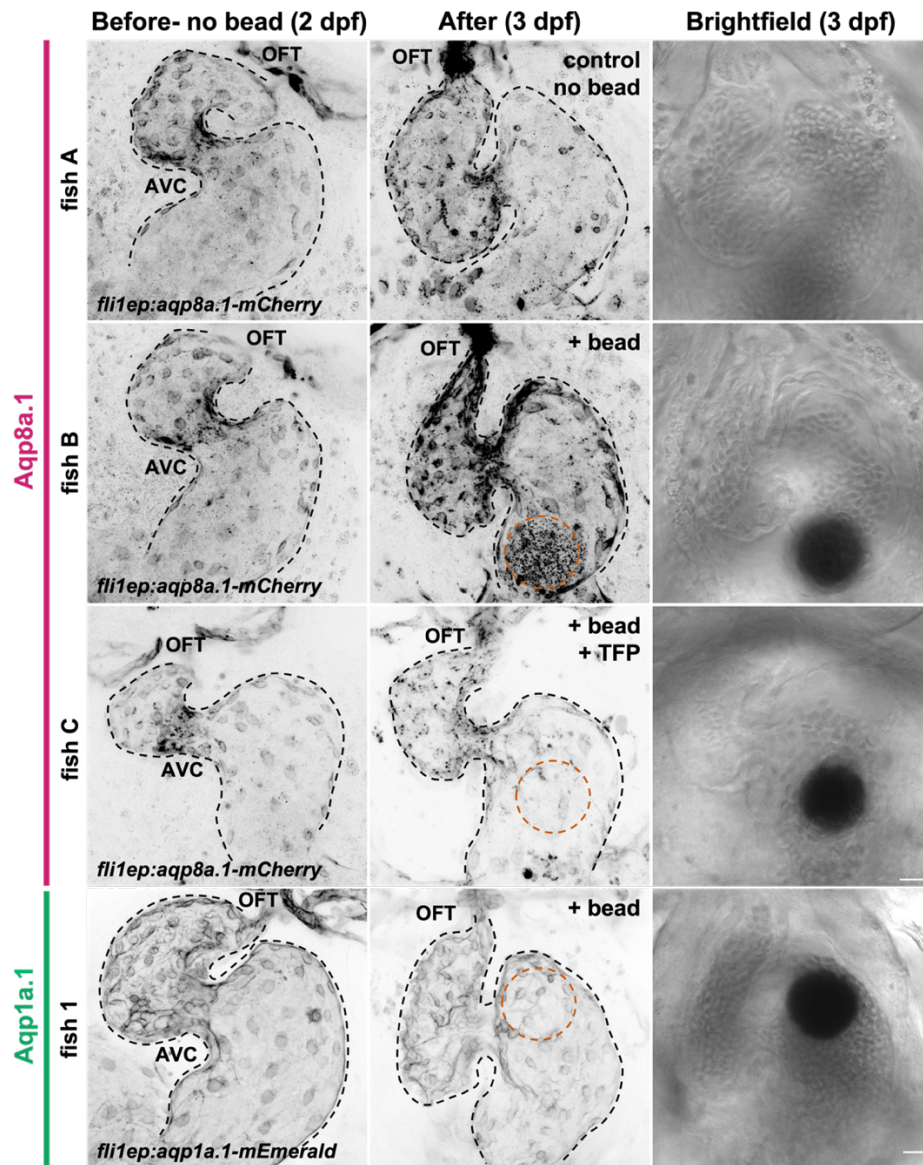


Figure 44 | Aqp8a.1 localisation is regulated by CaM activity. Confocal images of hearts of *Tg(fli1ep:aqp8a.1-mCherry)* (fish A, B and C) and *Tg(fli1ep:aqp1a.1-mEmerald)* (fish 1) zebrafish at 2 dpf, prior to bead grafting, and at 3 dpf under the following conditions: fish A without a bead (N= 5), fish B with a grafted bead (N= 4), fish C with a grafted bead in the presence of TFP (N= 4), and fish1 with a grafted bead (N= 4). Changes in protein localisation are observed in the case of *Tg(fli1ep:aqp8a.1-mCherry)* zebrafish when a bead is inserted into their atrium (fish B). Treatment with TFP not only blocks the anticipated Aqp8a.1 localisation changes induced by the grafted bead but also prevents the enrichment of the protein at the AVC (fish C). No changes in localisation are observed *Tg(fli1ep:aqp1a.1-mEmerald)* zebrafish when a bead is inserted in their atrium (fish 1). Black dotted lines outline the endocardium. Orange dotted lines indicate the bead. Scale bar: 20 µm.

2.4.4 The presence of a CaM-binding site drives Aqp8a.1 localisation changes

The activity of CaM and the presence of the CaM-binding domain in Aqp8a.1 emerge as important factors in modulating the localisation dynamics of the water channel. To further investigate the significance of the CaM-binding site and confirm its role in Aqp8a.1 motility, we undertook a rigorous site-directed mutagenesis approach involving both Aqp8a.1 and Aqp1a.1.

Specifically, we engineered a mutated version of Aqp8a.1 lacking the 26-amino acid CaM-binding domain spanning from residues 235 to 260. Concurrently, we generated a mutated version of Aqp1a.1 by replacing its last 26 amino acids with the corresponding CaM-binding site sequence from Aqp8a.1 (**Figure 45**). Given that both Aqp1a.1 and Aqp8a.1 consist of 260 amino acids, we precisely substituted residues 235 to 260 in Aqp1a.1. Our primary objective was to determine whether the absence or presence of the CaM-binding site alone can induce alterations in the localisation patterns of Aqp8a.1 and Aqp1a.1, respectively, providing insights into the regulatory role of this domain.

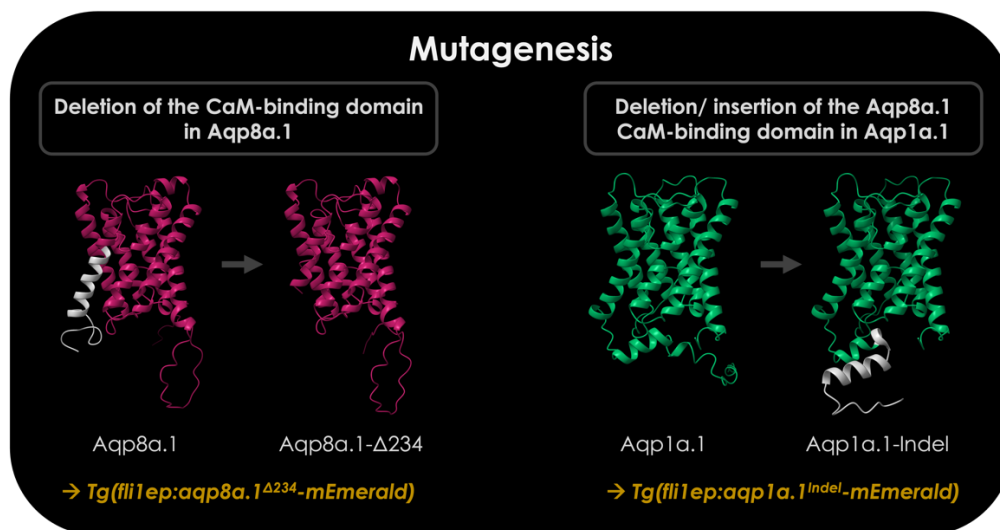


Figure 45 | Schematic illustrating the site-directed mutagenesis strategy. Structural schematics were predicted using AlphaFold (Jumper et al., 2021) and indicate the presence of a putative CaM-binding site (depicted in white) in the wild-type version of Aqp8a.1 and the mutated version of Aqp1a.1.

To examine the localisation of the mutated Aqp8a.1- Δ 234 and Aqp1a.1-Indel proteins, we generated the zebrafish transgenic lines *Tg(fli1ep:aqp8a.1 Δ 234-mEmerald)* and *Tg(fli1ep:aqp1a.1^{Indel}-mEmerald)*. Following the methodology described earlier (refer to 2.3.1 *Aqp1a.1 and Aqp8a.1 in EdC volume regulation and AV valve formation*), we arrested hearts overexpressing Aqp8a.1- Δ 234 and Aqp1a.1-Indel using BDM and imaged them at 48 hpf. To complement this analysis and enhance the mEmerald signal, we performed IF targeting GFP, and thereby mEmerald, in *Tg(fli1ep:aqp8a.1 Δ 234-mEmerald)* zebrafish at 48 hpf. Strikingly, our analysis revealed that Aqp8a.1- Δ 234 was primarily localised to the perinuclear region of EdCs, with no discernible enrichment of the protein at the AVC. Furthermore, no protein aggregates were formed (**Figures 46, 47 & 48**). Intriguingly, Aqp1a.1-Indel was mainly localised to the perinuclear region and the cell membrane, with the formation of aggregates, similarly to wild-type Aqp8a.1. Additionally, the mutated protein was notably enriched at the AVC compared to wild-type Aqp1a.1 (**Figures 46 & 48**). These results suggest that the presence of the CaM-binding site alone can substantially modify the localisation patterns of Aqp8a.1 and Aqp1a.1.

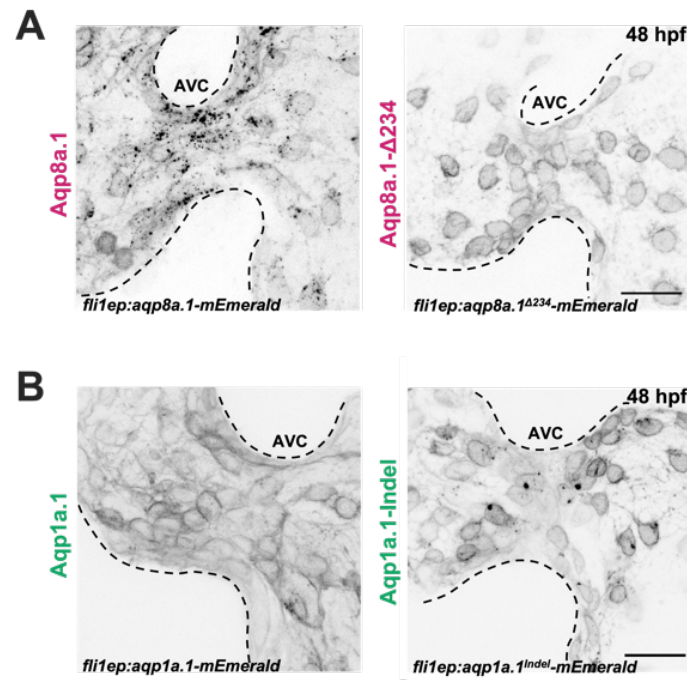


Figure 46 | Aqp8a.1-Δ234 and Aqp1a.1-Indel exhibit altered localisation compared to their wild-type versions. Maximum projections of (A) *Tg(fli1ep:aqp8a.1-mEmerald)* (N= 5) and *Tg(fli1ep:aqp8a.1^{Δ234}-mEmerald)* (N= 6), and (B) *Tg(fli1ep:aqp1a.1-mEmerald)* (N= 6) and *Tg(fli1ep:aqp1a.1^{Indel}-mEmerald)* (N= 5) embryonic hearts at 48 hpf. Aqp8a.1-Δ234 exhibits altered localisation compared to wild-type Aqp8a.1, primarily concentrated to the perinuclear region of the EdCs, without the formation of protein aggregates. Similarly, Aqp1a.1-Indel displays modified localisation, mainly localised to the perinuclear region and the cell membrane, with the formation of aggregates, akin to wild-type Aqp8a.1. Black dotted lines outline the endocardium. Scale bar: 20 μm.

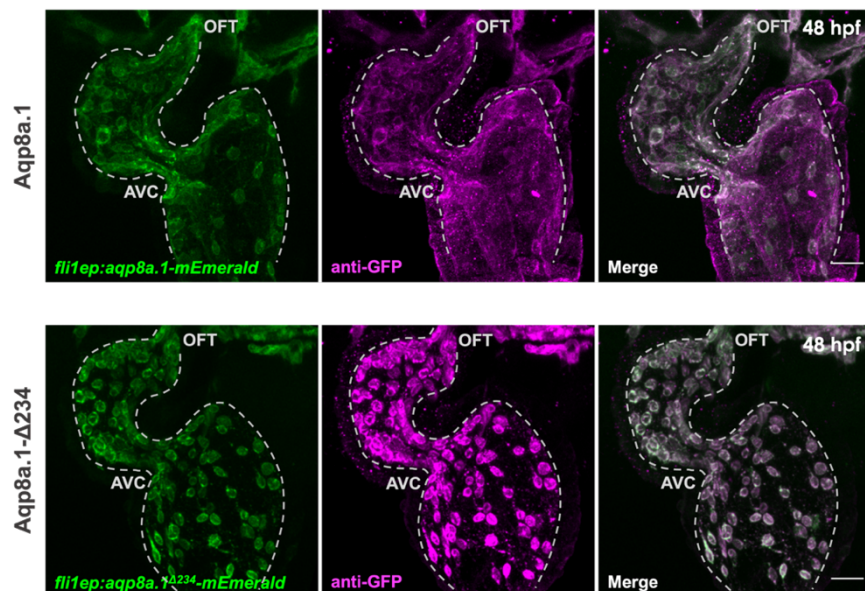


Figure 47 | Aqp8a.1-Δ234 exhibits altered localisation compared to wild-type Aqp8a.1, primarily concentrated to the perinuclear region of the EdCs. Maximum projections of GFP- immunostained embryonic hearts of *Tg(fli1ep:aqp8a.1-mEmerald)* (N= 11) and *Tg(fli1ep:aqp8a.1^{Δ234}-mEmerald)* (N= 8) zebrafish embryos at 48 hpf. White dotted lines outline the endocardium. Scale bar: 20 μm.

To further analyse the impact of the absence or presence of the CaM-binding site on the localisation of Aqp8a.1 and Aqp1a.1, we aimed to investigate changes in the localisation of Aqp8a.1- $\Delta 234$ and Aqp1a.1-Indel upon increased mechanical forces and calcium influx. For this purpose, we employed the bead insertion approach in *Tg(fli1ep:aqp8a.1 $\Delta 234$ -mEmerald)* and *Tg(fli1ep:aqp1a.1^{Indel}-mEmerald)* zebrafish, following the methodology described in 2.4.2 *Aqp8a.1 localisation is controlled by mechanical forces*. Notably, we observed no localisation changes in the presence of the bead for Aqp8a.1- $\Delta 234$, similarly to wild-type Aqp1a.1. However, in the case of Aqp1a.1-Indel, distinct changes in the protein's localisation were evident across the developing heart, particularly noticeable at the AVC and the ventricle, mirroring observations made with wild-type Aqp8a.1 (**Figure 48**).

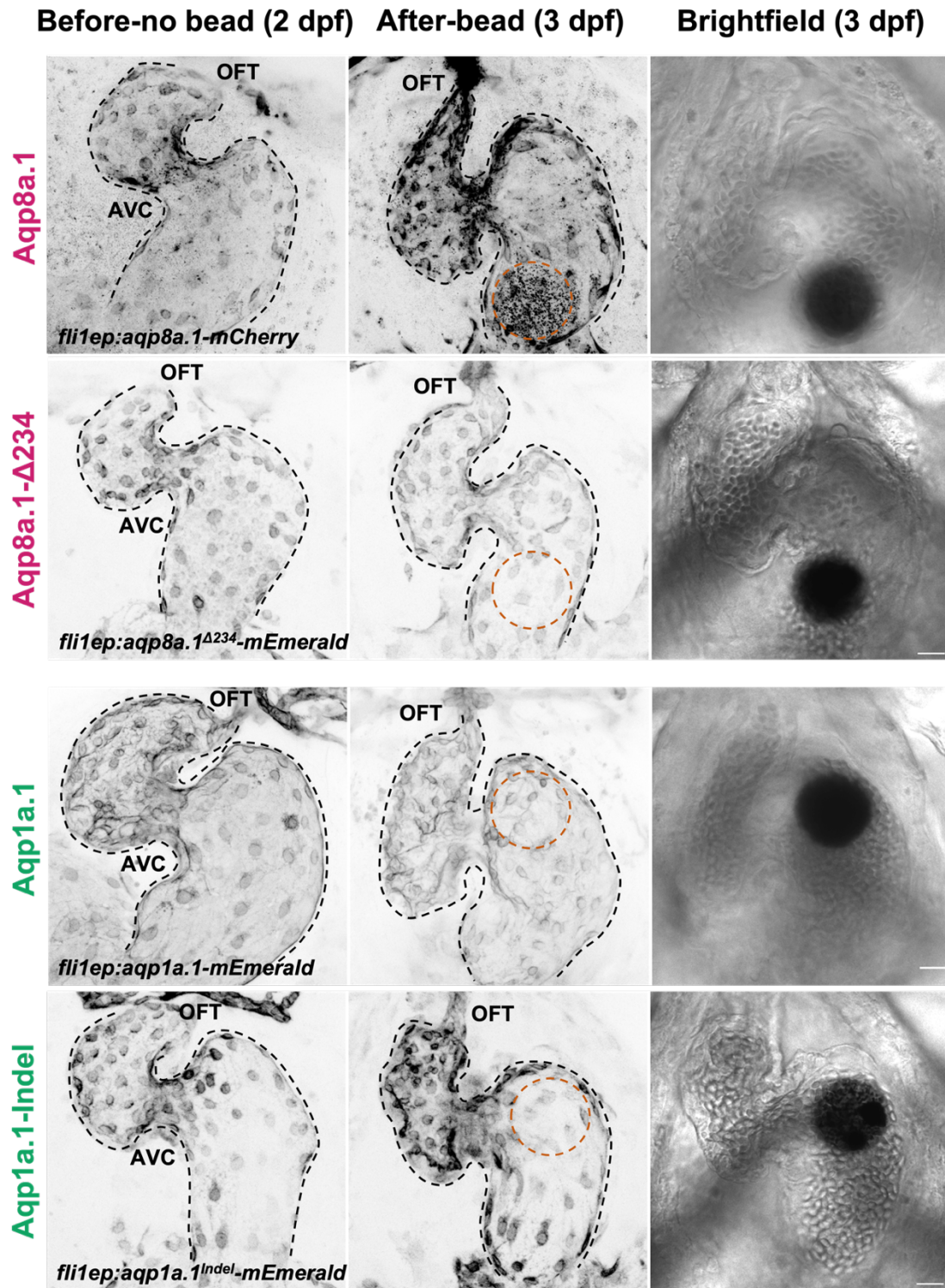


Figure 48 | The presence of a CaM-binding site drives Aqp8a.1 and Aqp1a.1 localisation. Confocal images of *Tg(fli1ep:aqp8a.1-mCherry)* (N= 4), *Tg(fli1ep:aqp8a.1^{Δ234}-mEmerald)* (N= 4), *Tg(fli1ep:aqp1a.1-mEmerald)* (N= 4) and *Tg(fli1ep:aqp1a.1^{Indel}-mEmerald)* (N= 3) embryonic hearts at 2 dpf, prior to bead grafting, and at 3 dpf, with a bead grafted in the atrium. In the presence of the bead, no localisation changes were observed for Aqp8a.1-Δ234, akin to wild-type Aqp1a.1. However, distinct changes in the protein's localisation were evident across the developing heart for Aqp1a.1-Indel, particularly noticeable at the AVC and the ventricle, mirroring observations made with wild-type Aqp8a.1. Black dotted lines outline the endocardium. Orange dotted lines indicate the bead. Scale bar: 20 μm.

Overall, these findings underscore the pivotal role of the CaM-binding site in modulating the localisation dynamics of Aqp8a.1 and Aqp1a.1 in response to mechanical forces and calcium influx.

2.5 F-actin remodelling is essential for AVC EdC cell volume regulation

2.5.1 F-actin remodelling is absent in AVC EdCs upon Aqp8a.1 loss

The dynamic regulation of AVC EdC volume decrease during early valve development is associated with F-actin remodelling and active cell contractility, specifically in the cells responsible for forming the AV valve (Vignes et al., 2022).

Therefore, we aimed to examine potential alterations in the organisation of F-actin in *aqp8a.1* mutant embryos, which exhibit increased AVC EdC volume and ensuing AV valve defects. To visualise F-actin distribution, we conducted phalloidin staining at 48 hpf. Intriguingly, our analysis unveiled lack of F-actin remodelling in EdCs located at the AV valve-forming region upon the loss of Aqp8a.1 (**Figure 49**). These findings indicate a correlation between the absence of Aqp8a.1 and F-actin remodelling, a key feature of AVC EdC volume reduction.

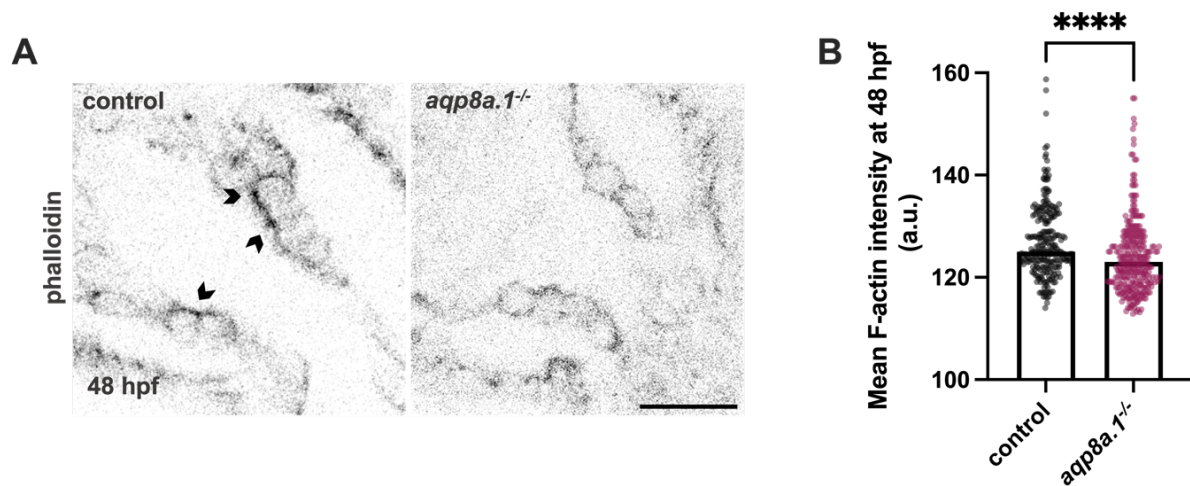


Figure 49 | F-actin remodelling is absent in EdCs located at the AV valve-forming region upon loss of Aqp8a.1. (A) Single z-plane immunofluorescence images of phalloidin staining in control (N= 7) and *aqp8a.1* mutant (N= 8) embryos. Black arrows indicate EdCs of the AVC with enrichment in F-actin. Scale bar: 20 μ m. (B) Quantification of the F-actin fluorescent intensity based on the data in (A). a.u.: arbitrary units. Statistical analysis was performed using a two-tailed unpaired t-test.

2.5.2 Marcksl1 is essential for F-actin remodelling in zebrafish EdCs

The intricate process of tissue morphogenesis is orchestrated by actomyosin networks and actin crosslinking proteins (Collinet and Lecuit, 2021, Krueger et al., 2019, Munjal and Lecuit, 2014). While a critical interplay between actin dynamics and AV valve development is implied (Vignes et al., 2022), the necessity of F-actin remodelling and the implication of actin crosslinkers, such as Marcksl1, in EdC volume modulation have not been investigated.

In zebrafish, *marcksl1a* and *marcksl1b* are paralogues of Marcksl1, encoding for a crosslinking protein that bundles and stabilises F-actin (Björkblom et al., 2012, Wohnsland et al., 2000). Marcksl1 is enriched at the apical membrane of endothelial cells and regulates cortical actin organisation. Its expression has been associated with the regulation of endothelial cell size and blood vessel diameter (Kondrychyn et al., 2020).

Interestingly, RNA sequencing data from the Vermot lab reveal that *marcksl1a* and *marcksl1b* are expressed in the embryonic heart during early stages of valvulogenesis (unpublished). Hence, we aimed to assess the potential role of Marcksl1 in F-actin remodelling and its impact on in AVC EdC volume regulation.

To test whether Marcksl1 governs F-actin distribution during AV valve formation, we used a zebrafish mutant line harbouring mutations in both *marcksl1a* (*rk23*) and *marcksl1b* (*rk24*) (Kondrychyn et al., 2020) and evaluated F-actin distribution through phalloidin staining at 48 hpf. Our findings revealed a significant impairment in F-actin remodelling in the absence of Marcksl1, with no discernible F-actin enrichment observed in the AVC EdCs compared to control embryos (**Figure 50**). These results indicate that Marcksl1 is required for the organisation of F-actin during cardiac valve formation.

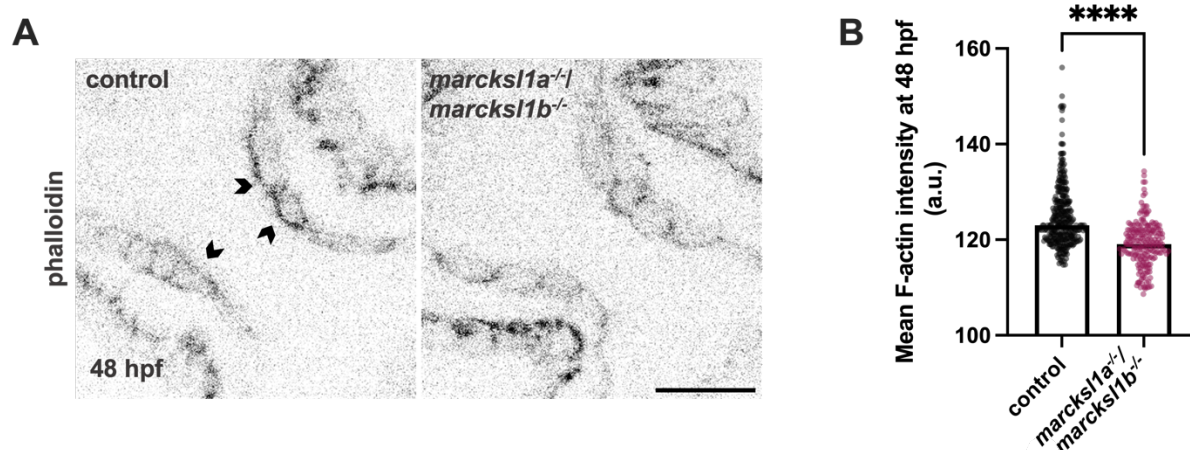


Figure 50 | F-actin remodelling is absent in EdCs located at the AV valve-forming region upon loss of Marcksl1. (A) Single z-plane immunofluorescence images of phalloidin staining in control (N= 8) and *marcksl1a;marcksl1b* mutant (N= 5) embryos. Black arrows indicate EdCs of the AVC with enrichment in F-actin. Scale bar: 20 μ m. (B) Quantification of the F-actin fluorescent intensity based on the data in (A). a.u.: arbitrary units. Statistical analysis was performed using a two-tailed unpaired t-test.

2.5.3 Marcksl1 is required for AVC EdC cell volume regulation

To investigate the implication of Marcksl1 in EdC volume regulation, we set out to quantify the EdC volume in *marcksl1a;marcksl1b* mutants. Following the methodology outlined earlier (see 2.1.1 *Piezo1 is involved in EdC cell volume regulation*), we performed IF targeting Fli-1 and subsequently measured the EdC volume in the absence of Marcksl1 at 48 hpf. Remarkably, we found a significant increase in AVC EdC volume compared to control embryos (**Figure 51A**). These data suggest that Marcksl1 and, thereby F-actin remodelling, are essential for AVC EdC volume reduction.

To further investigate the impact of the absence of Marcksl1 on valve morphogenesis, we examined the heart morphology of *marcksl1a* and *marcksl1b* mutants at 80 hpf, after conducting IF against Fli-1. This analysis showed that the AV valve develops normally (**Figure 51B**), suggesting that the loss of Marcksl1 does not influence the formation of the valve.

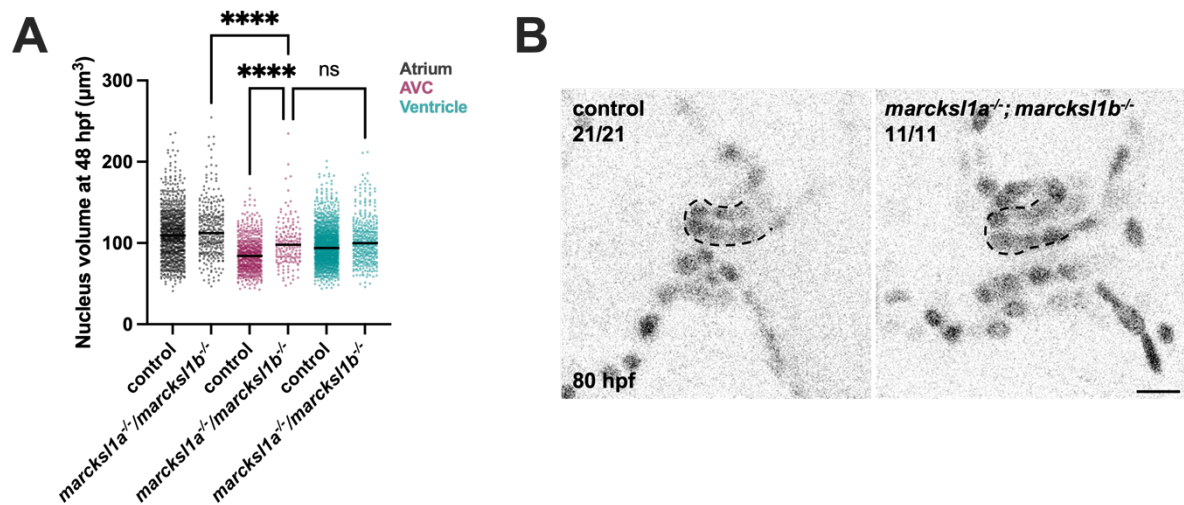


Figure 51 | Marcks1 is required for AVC EdC volume decrease. (A) Quantification of the nucleus volume in control (N= 16) and *marcks1a;marcks1b* mutant (N= 5) embryos at 48 hpf. Statistical analysis was performed using a two-tailed Mann-Whitney test. (B) Single z-plane images of the superior AV valve leaflet (EdCs immunostained for Fli1) of control and *marcks1a;marcks1b* mutant embryos at 80 hpf, demonstrating normal AV valve elongation in both groups. Black dotted lines outline the superior AV valve leaflet. Scale bar: 10 μm .

Chapter 3. Discussion

3.1 Key Findings

Amongst the mechanisms implicated in tissue development, the regulation of cell volume emerges as a pivotal modulator of morphogenesis. In zebrafish, EdC volume decrease represents a crucial developmental step for AV heart valve formation and marks the onset of valvulogenesis (Vignes et al., 2022). However, the modulation of EdC volume changes remained unknown.

During my PhD, I aimed to unravel cellular and molecular mechanisms underlying EdC volume regulation in zebrafish AV valve development. Through a comprehensive series of experiments, I investigated the roles of Piezo ion channels, Notch signalling, endocardial-expressed aquaporins, F-actin remodelling, and actin crosslinkers in controlling AVC EdC volume. Leveraging advanced techniques such as live imaging, deep learning-based image analysis, generation of multiple transgenic zebrafish lines, analysis of mutant lines, microsurgery for bead implantation in the beating zebrafish heart, qualitative and quantitative *in situ* hybridisation approaches, and immunofluorescence assays, my research led to critical insights that address significant gaps in our understanding of EdC volume modulation and, consequently, cardiac valve morphogenesis.

In my PhD thesis, I demonstrate that the interplay of the mechanosensitive ion channel Piezo1, the receptor Notch1b and the Aqp8a.1 water channel orchestrates EdC volume reduction. I show that Aqp8a.1 is required for endocardial cell volume decrease, and *aqp8a.1* mutant larvae display heart valve defects, altered cell polarity and absence of F-actin remodelling. Furthermore, I demonstrate that *aqp8a.1* expression is regulated by Notch signalling. Mechanistically,

overexpression of Piezo1 using the genetically encoded Piezo1 biosensor GenEPI leads to downregulation of *notch1b* and subsequently *aqp8a.1*.

At the protein level, the Ca^{2+} -binding CaM protein can directly bind to the Aqp8a.1 carboxyl terminus, driving Aqp8a.1 localisation. Strikingly, I find that the activity of CaM and the presence of a putative CaM-binding domain in Aqp8a.1 play a central role in determining the localisation dynamics of the water channel in response to mechanical forces and, thereby, calcium influx.

In parallel with these mechanisms, F-actin remodelling arises as an essential event for AVC EdC cell volume reduction. Specifically, the actin crosslinking protein Marcksl1 is required for the organisation of F-actin in AVC EdCs and the regulation of EdC volume during cardiac valve formation.

Altogether, my work identifies new effectors of EdC volume changes and heart morphogenesis (**Figure 52**), elucidating the complexities of embryonic development and significantly advancing our fundamental knowledge on early organogenesis.

3.2 Piezo ion channels in EdC volume regulation

Recent studies have identified Piezo1 and Piezo2a as critical factors for the formation of functional AV (Juan et al., 2023) and OFT (Duchemin et al., 2019, Faucherre et al., 2020) valves during late developmental stages. Nevertheless, their potential role throughout early stages, especially when the morphogenesis of the AV valve initiates, remained unexplored.

To address this gap, I investigated the implication of Piezo1 and Piezo2a in early valvulogenesis, by first examining their expression patterns in the developing zebrafish heart. Specifically, I focused on 36 hpf, which coincides with the onset of AVC EdC volume decrease, and on 48 hpf, just prior to the extension of basal protrusions by EdCs into the cardiac jelly. I found that both *piezo1* and *piezo2a* are expressed in the embryonic heart at these time points. These findings are supported by data obtained from single-cell RNA sequencing conducted by the Stainier lab (data not shown, personal communication), confirming the endocardial expression of *piezo1* and *piezo2a*. Future experiments using RNAscope assays will validate the presence of Piezo1 and Piezo2a within the endocardial layer and will provide quantitative readouts of the distribution of the mRNAs across the different regions of the heart.

To analyse the impact of Piezo1 on AVC EdC volume decrease, I used a zebrafish Piezo1 overexpression line (Yaganoglu et al., 2023). Interestingly, I demonstrated that overexpression of Piezo1 affects EdC volume and leads to a significant increase in AVC EdC volume. Additionally, I used zebrafish mutant lines for *piezo1* and *piezo2a* (Juan et al., 2023), and found that AVC EdC volume is further decreased in *piezo2a* single mutants compared to wild-type siblings, yet the difference is not statistically significant. These results are particularly intriguing as, first, *piezo* mutants tend to exhibit the opposite phenotype to zebrafish overexpressing Piezo1, and second, they could imply that the absence of a significant reduction in the EdC volume in single *piezo* mutants could be due to genetic compensation between the two channels (El-Brolosy et al., 2019, El-Brolosy and Stainier, 2017). Therefore, prioritising future work on the study of double *piezo1;piezo2a* mutants will help exclude this possibility and will shed light on these mechanisms.

Moreover, Piezo1 and Piezo2a ion channels have been suggested to regulate the expression of genes essential for valve development (Duchemin et al., 2019, Juan et al., 2023). Therefore, I sought to investigate whether Piezo1 overexpression impacts the expression of genes of interest. Notably, I showed that the mRNA levels of *notch1b* and *aqp8a.1* are reduced under this condition at 36 hpf. These findings indicated that the overexpression of Piezo1 downregulates the expression of *notch1b* and *aqp8a.1* during a critical stage of AVC EdC volume regulation, potentially accounting for the observed increase in AVC EdC volume upon Piezo1 overexpression.

The downregulation of *notch1b* following overexpression of Piezo1 is consistent with previous findings. Specifically, Piezo1 has been shown to tightly regulate the expression levels of *klf2a* and modulate both Klf2 and Notch activity in the OFT endothelium. Characteristically, in *piezo1* mutants, Klf2a is upregulated (Duchemin et al., 2019). Similarly, *pkd* genes cooperate to repress *klf2a* and *klf2b* during AV valve formation, and in *pkd* mutants, Klf2a is upregulated (Juan et al., 2023). These observations underscore the necessity for strict regulation of the expression of these transcriptional factors, as higher levels negatively impact AV valve development; both *piezo1* and *pkd* mutants exhibit valve defects (Juan et al., 2023). In the context of EdC volume modulation, Klf2a and Klf2b are not involved in AVC EdC volume control (Vignes et al., 2022); hence, Notch1b may be a key modulator of this process. In addition, it has been reported that *notch1b* expression remains unaffected in *trpp2;trpv4* double mutants, indicating that *notch1b* is not regulated by TRP channels (Vignes et al., 2022). Given the mechanosensitivity of *notch1b* expression (Vignes et al., 2022, Vermot et al., 2009), it is therefore plausible that Piezo channels may be involved in its modulation. In the future, studies investigating the expression of *notch1b* in *piezo1;piezo2a* mutants will complement this analysis. A remarkable aspect to note is that at the protein level, Piezo1 has been suggested

to trigger Notch1 cleavage in response to calcium influx, subsequently driving the expression of target genes in the hepatic vasculature of mice (Caolo et al., 2020). This highlights the intricate interplay between Piezo1 signalling and Notch pathway activation and further research on this level will shed light on the regulatory mechanisms governing development.

Overexpression of Piezo1 leads to downregulation of *notch1b*; however, the intermediates of this signalling pathway remained unclear. Recently, the Ca^{2+} /calmodulin-dependent protein kinase Camk2g1 was identified as an intermediate effector of Pkd response upstream of *klf2a* expression, during zebrafish AV valve formation (Juan et al., 2023). In my study, findings showed that *notch1b* expression remains unaffected upon overexpression of a constitutively active (CA) variant of Camk2g1 at 36 hpf, suggesting that its regulation is independent of Camk2g1 activity. These results could be explained by considering that morphogenetic programmes may rely on a multitude of mechanisms and effectors to ensure physiological organogenesis. This redundancy acts as a safeguard mechanism in case of disruption in one of the pathways, akin to a back-up system, and highlights that not all factors involved in developmental processes necessarily depend on the same mechanisms.

Piezo1 has also been linked to the cell volume homeostasis of red blood cells in zebrafish (Faucherre et al., 2014) and mouse models (Cahalan et al., 2015). Notably, in blood isolated from wild-type mice, activation of Piezo1 through Yoda1, a chemical activator of Piezo1, leads to calcium influx, resulting in the dehydration of red blood cells. The reduction in cell volume is mediated by a downstream ion channel that facilitates the efflux of potassium ions and water molecules from the cells, directly implicating Piezo1 signalling in the control of cell volume (Cahalan et al., 2015). This cell volume regulatory process becomes particularly intriguing when

considered alongside AVC EdC volume modulation in zebrafish. In this context, Piezo1 is activated by mechanical forces generated by the flow of blood within the zebrafish heart, causing Ca^{2+} influx. This tightly regulates the expression levels of Notch1b, which subsequently activates *aqp8a.1*. The localised expression of the Aqp8a.1 water channel likely promotes water efflux, initiating the process of AVC EdC volume decrease, which is crucial for valve formation. Thus, it becomes evident that the two mechanisms involving Piezo1 in cell volume regulation in red blood cells and the endocardium could be considered aligned.

Studies on both *piezo1* mouse mutants and *piezo1* zebrafish morphants demonstrate that the absence of Piezo1 results in red blood cell swelling (Cahalan et al., 2015, Faucherre et al., 2014). Interestingly, these data seemingly contradict my results on Piezo1 overexpression, which also leads to increased AVC EdC volume in zebrafish. However, at this point, it is important to consider the distinct microenvironments of *in vitro* treated erythrocytes (Cahalan et al., 2015) or blood vessels (Faucherre et al., 2014) and the embryonic heart, which may be responsible for the observed differences. It is essential to acknowledge that complex physiological interactions may result in different mechanisms being implicated under varying conditions, thereby highlighting the need for further investigation into the context-dependent roles of Piezo1 in diverse biological settings.

3.3 Notch signalling in AVC EdC volume decrease

Zebrafish heart development relies on functional Notch signalling, and disruption of the pathway leads to defective AV valve formation (Timmerman et al., 2004). Notably, *notch1b* is prominently expressed in the endocardium during the initiation of AV valve morphogenesis (Vermot et al., 2009, Milan et al., 2006), and Notch signalling is crucial for modulating AVC EdC volume (Vignes et al., 2022). However, the role of

Notch1b and the Notch cascade in this process, along with their interaction with other factors implicated in AVC EdC volume reduction, had not been explored.

Interestingly, Notch signalling has been identified to directly control the transcription of an aquaporin family member, specifically AQP3, during human keratinocyte differentiation (Guo et al., 2013). Given this insight, and since overexpression of Piezo1 downregulates the expression of both *notch1b* and *aqp8a.1*, I sought to examine whether *aqp8a.1* expression in zebrafish is downstream of Notch1b and is similarly regulated by Notch signalling. Remarkably, upon perturbation of the Notch pathway using DAPT, I found a significant downregulation and absence of *aqp8a.1* expression at 36 hpf. These results indicate that the expression of *aqp8a.1* is indeed modulated by Notch signalling and could suggest a potential conserved role for Notch signalling in the regulation of aquaporin expression across different biological contexts.

3.4 Aqp1a.1 and Aqp8a.1 in EdC volume modulation

Aqp1a.1 and *aqp8a.1* are expressed in the endocardium during the onset of AV valve morphogenesis, and their mRNA distribution is enriched in the AVC (Vignes et al., 2022). This aspect is particularly interesting, especially within the context of EdC volume regulation. A mechanism for reducing cell volume involves reducing water content. Aquaporins have the ability to enhance membrane permeability (Ibata et al., 2011, Mola et al., 2016) and promote regulatory volume changes based on osmotic and hydrostatic gradients across the cell membrane (Mola et al., 2016). Given the negatively charged ECM, which directly interacts with EdCs and can attract water, the localised expression of aquaporins (Vignes et al., 2022) suggests that the two channels may play a pivotal role in regulating AV valve formation by facilitating

water efflux and thereby controlling AVC EdC volume decrease. Yet, their implication in this process remained elusive.

To investigate the potential role of Aqp1a.1 and Aqp8a.1 in EdC volume regulation, I used a zebrafish mutant line harbouring mutations in both *aqp1a.1* and *aqp8a.1* (unpublished data from the Phng lab). Remarkably, I found a significant increase in AVC EdC volume in the absence of the two proteins. These data suggest that Aqp1a.1 and Aqp8a.1 are essential for AVC EdC volume reduction.

Since the previous findings of my work indicated that the expression of *aqp8a.1*, but not *aqp1a.1*, is affected by Piezo1 overexpression and Notch signalling disruption, both of which conditions lead to dysregulation of EdC volume, I focused on examining *aqp8a.1* single mutants. Strikingly, I demonstrated a significant increase in AVC EdC volume upon loss of Aqp8a.1, similarly to the double mutants. These results conclusively reveal that Aqp8a.1 alone is sufficient to mediate AVC EdC volume reduction and promote EdC volume changes. Moreover, analysis of the *aqp8a.1* mutants showed altered cell polarity and AV valve elongation defects, suggesting that Aqp8a.1 is directly involved in AV valve morphogenesis.

Considering the pivotal role of Aqp8a.1 in promoting EdC volume decrease and AV valve morphogenesis, my subsequent objective was to investigate the localisation of this water channel within the embryonic zebrafish heart. To accomplish this and given the lack of effective antibodies for zebrafish, I established overexpression lines for Aqp8a.1, namely *Tg(fli1ep:aqp8a.1-mEmerald)* and *Tg(fli1ep:aqp8a.1-mCherry)*. Analysis of the transgenic zebrafish revealed that Aqp8a.1 is primarily localised to the plasma membrane and the perinuclear region of EdCs. Additionally, I observed numerous aggregates of the protein. To confirm the localisation of Aqp8a.1, I conducted multiple colocalisation assays using known markers of the cell

membrane and the nucleus, which validated the initial results. In addition, in order to examine the nature of the observed protein aggregates, colocalisation assays were performed using known markers of the mitochondria and endosomes, where no colocalisation was observed (data not shown, personal communication- Phng lab). Thus, it remains unclear what these aggregates represent.

Interestingly, AQP8 has been reported to be primarily located in intracellular vesicles (Gradilone et al., 2003, García et al., 2001). Multiple studies suggest its subcellular translocation from the cytoplasm to the plasma membrane in rat hepatocytes (Gradilone et al., 2003, García et al., 2001), the human colon (Lin et al., 2023) and the rat gastrointestinal tract (Calamita et al., 2001), a mechanism controlling the abundance of aquaporins on the cell surface (Markou et al., 2022). Thus, Aqp8a.1 aggregates in the zebrafish heart may represent intracellular vesicles potentially regulating membrane permeability.

To determine the identity and functional significance of the observed protein aggregates, several future experimental approaches and their combination will be necessary. These include: (i) Electron Microscopy (EM) to visualise the ultrastructure of the protein aggregates at high resolution. Specifically, Correlative Light and Electron Microscopy (CLEM), which enables the combination of confocal microscopy with EM for the characterisation of biological samples (Goetz et al., 2015, Goetz et al., 2014), will be appropriate in this study. To facilitate this, I have initiated the generation of a zebrafish transgenic line *Tg(fli1ep:aqp8a.1-mEos4b)*. Notably, mEos4b is a photoconvertible red fluorescent protein with strong resistance of fluorescence and photoconversion to osmium tetroxide (OsO₄) (Paez-Segala et al., 2015), a common fixative used to preserve cellular ultrastructure for EM (Palade, 1952). (ii) Photoconversion to monitor the dynamics and behaviour of the protein aggregates. This approach can provide valuable insights into whether

the aggregates are static or dynamic structures, thereby offering clues about their functional relevance. The *Tg(fli1ep:aqp8a.1-mEos4b)* line will be apt for this purpose.

Intriguingly, despite Aqp8a.1 being expressed under the *fli1* promoter in the *Tg(fli1ep:aqp8a.1-mEmerald)* and *Tg(fli1ep:aqp8a.1-mCherry)* lines, which would typically result in an even distribution of the protein within EdCs, Aqp8a.1 is evidently enriched at the AVC, where mechanical forces and calcium levels are notably elevated at the AVC (Fukui et al., 2021, Juan et al., 2023). This suggests that the localisation of the Aqp8a.1 may be influenced by shear stress and calcium influx. More precisely, given the mechanism of AQP8 translocation in other systems (Gradilone et al., 2003, García et al., 2001, Lin et al., 2023, Calamita et al., 2001), I hypothesised that shear stress and incoming calcium stabilise Aqp8a.1 by enhancing its incorporation into the plasma membrane.

To examine whether mechanical stimuli and increased intracellular calcium levels can induce changes in Aqp8a.1 localisation, I employed a bead setup. This method involves the precise surgical insertion of a bead into the atrium of the beating heart of anaesthetised zebrafish, resulting in the amplification of the mechanical forces experienced by EdCs, thereby directly triggering calcium influx (Fukui et al., 2021). By grafting a bead in the heart of fish overexpressing Aqp8a.1, I observed evident changes in the protein's localisation throughout the developing heart, particularly at the AVC and the ventricle. These results indicate that the localisation of Aqp8a.1 is governed by both mechanical forces and intracellular calcium levels.

Following these findings, I aimed to further understand the mechanism underlying the localisation changes of Aqp8a.1. To this end, I directed my focus towards the Ca^{2+} -binding protein Calmodulin (CaM). CaM can directly bind to various

aquaporins (Reichow et al., 2013, Kitchen et al., 2020, Rabaud et al., 2009, Reichow and Gonen, 2008) and regulate their cell-membrane localisation through vesicular trafficking (Kitchen et al., 2020), in response to intracellular Ca^{2+} concentration changes (Reichow et al., 2013, Vogel, 1994).

Using the Calmodulin Target Database (Yap et al., 2000), I identified a putative CaM-binding site at the carboxyl terminus of Aqp8a.1, spanning residues 235 to 260, suggesting that the water channel can directly interact with CaM. Importantly, within this site, I identified a potential CaM-binding motif characterised by three leucine residues forming a hydrophobic surface, with charged residues on the other side, which is a typical characteristic of CaM-binding helices (Tidow and Nissen, 2013).

To investigate whether CaM is involved in the regulation of Aqp8a.1 localisation, I employed the same bead insertion approach while inhibiting CaM using TFP. Remarkably, I found that inhibition of CaM not only blocks the expected Aqp8a.1 localisation changes induced by the grafted bead but also prevents the enrichment of the protein at the AVC. These data suggest that Aqp8a.1 localisation is regulated by CaM activity. In order to determine whether the three leucine residues forming the characteristic potential CaM-binding motif within the CaM-binding site are indeed essential for the interaction of Aqp8a.1 with CaM, site-directed mutagenesis leading to the generation of a mutated version of Aqp8a.1, wherein the three leucine residues are replaced by alanine, will be imperative. Investigation of the phenotype of fish overexpressing this altered Aqp8a.1 fused to a fluorescent protein under the *fli1* promoter will reveal the importance of the leucine residues.

To complement this analysis, I studied Aqp1a.1, which lacks a predicted CaM-binding domain and is uniformly distributed throughout the endocardium without the formation of protein aggregates in *Tg(fli1ep:aqp1a.1-mEmerald)* zebrafish.

Significantly, when a bead was inserted in the heart of fish overexpressing Aqp1a.1, no localisation changes were observed. These findings provide additional support for the hypothesis that the presence of the CaM-binding site in Aqp8a.1 is crucial for the protein's localisation dynamics.

To further investigate the significance of the CaM-binding site and gain insights into the regulatory role of this domain, I employed a site-directed mutagenesis approach involving both Aqp8a.1 and Aqp1a.1. This led to the generation of Aqp8a.1- Δ 234, a mutated version of Aqp8a.1 lacking the 26-amino acid CaM-binding domain, and Aqp1a.1-Indel, a mutated version of Aqp1a.1 in which its last 26 amino acids are replaced by the corresponding CaM-binding site sequence from Aqp8a.1. By investigating the phenotype of fish overexpressing Aqp8a.1- Δ 234 and Aqp1a.1-Indel under the *fli1* promoter, a surprising discovery emerged: the localisation of the two proteins is reversed. Aqp1a.1-Indel now exhibits localisation similar to wild-type Aqp8a.1, while Aqp8a.1- Δ 234 adopts the localisation pattern of wild-type Aqp1a.1.

To examine changes in the localisation of Aqp8a.1- Δ 234 and Aqp1a.1-Indel under increased mechanical forces and calcium influx, I used the bead insertion method in *Tg(fli1ep:aqp8a.1 Δ 234-mEmerald)* and *Tg(fli1ep:aqp1a.1^{Indel}-mEmerald)* zebrafish. Remarkably, this analysis revealed that, in the presence of the grafted bead, the localisation of Aqp1a.1-Indel mirrors the changes observed in wild-type Aqp8a.1, whereas the localisation of Aqp8a.1- Δ 234 remains unaffected. These findings are particularly intriguing, as they demonstrate that altering 10% of the amino acid sequence of two distinct proteins can profoundly influence their behaviour, leading to dynamic subcellular relocalisation and therefore potential modulation of their functions.

In general, aquaporins may exhibit various intracellular localisations. While their primary location is within the plasma membrane, some aquaporins are also found within intracellular vesicles. Notably, they can translocate to the plasma membrane in response to external stimuli, such as hormones, hypotonicity, and hypoxia (Conner et al., 2012, Kitchen et al., 2020, Nielsen et al., 1993, Salman et al., 2017). As mentioned above, AQP8 has been suggested to be primarily located in intracellular vesicles (Gradilone et al., 2003, García et al., 2001) and its subcellular trafficking from the cytoplasm to the plasma membrane (Gradilone et al., 2003, García et al., 2001, Calamita et al., 2001, Lin et al., 2023) controls its abundance on the cell surface and thereby membrane permeability and water transport. Thus, I do reason that Aqp8a.1 aggregates in the zebrafish heart represent intracellular vesicles that translocate to the cell membrane in response to mechanical forces, thereby modulating water efflux and AVC EdC volume decrease. Moreover, the fact that Aqp8a.1 directly interacts with CaM, as indicated by the Calmodulin Target Database (Yap et al., 2000), which can regulate the cell-membrane localisation of aquaporins through vesicular trafficking (Kitchen et al., 2020), strengthens this hypothesis.

The observed changes in Aqp8a.1 localisation upon increased mechanical forces are challenging to defined at this point, primarily due to the lack of membrane markers. However, the introduction of these markers holds great promise, as they will help determine whether Aqp8a.1 translocates to the plasma membrane in response to an influx of Ca^{2+} and will facilitate quantification of the potential membrane integration percentage. Conducting future experiments that replicate the bead experiments with the inclusion of membrane markers will provide crucial insights.

Additionally, the observation that Aqp8a.1 localisation changes are particularly evident at the AVC and the ventricle is counterintuitive, given that changes would be expected mainly in cells in direct contact with the bead, in the atrium. However, it is

possible that the presence of the bead modifies the mechanical forces experienced by the EdCs throughout the heart, potentially by impacting blood flow dynamics. Regardless, it is known that mechanical forces are notably heightened at the AVC and the ventricle (Juan et al., 2023). Therefore, modelling the forces and their changes in the different regions of the heart due to the presence of the bead will shed light on this discrepancy. To address this, I am currently gathering data from beating zebrafish hearts overexpressing Aqp8a.1 in the presence of the bead, and in collaboration with the Yap lab, we are planning to model these forces comprehensively.

Moreover, it is essential to consider the timing of the post-bead grafting imaging, which is performed hours after the implantation of the bead in the atrium. This raises the possibility of intracellular signalling events taking place, potentially triggering the observed result. Remarkably, aquaporins have been found to localise in intracellular vesicles, forming multivesicular bodies (MVBs). MVBs can fuse with the plasma membrane to release these vesicles as exosomes, which can then integrate into other cells (Clarke-Bland et al., 2022). This mechanism could be at play in our system, accounting for the observed changes in Aqp8a.1 localisation.

In future investigations, exploring the physiological significance of subcellular relocalisation will elucidate the function of Aqp8a.1 and the complex regulatory mechanisms of cardiac morphogenesis. Specifically, it will be beneficial to explore the effects of these localisation alterations on AVC EdC volume regulation. This could involve examining EdC volume in zebrafish overexpressing Aqp8a.1 in the presence of a grafted bead. Additionally, generating a zebrafish mutant line for *aqp8a.1* with either a deletion in the CaM-binding site or mutations replacing the three leucine residues forming the characteristic potential CaM-binding motif within the CaM-binding site with alanine, and studying the impact on EdC volume, would provide

further insights into the importance of the interaction with CaM and the subsequent localisation changes in EdC volume regulation during AV valve morphogenesis.

Overall, this is the first time where the localisation of aquaporin water channels is shown to be regulated by CaM in response to mechanical forces. These findings shed light on how mechanical cues modulate the localisation and potentially activity of aquaporin channels, consequently impacting water balance regulation. Further investigation of the physiological relevance of these localisation changes will elucidate the significance of these events. These insights can open new avenues for developing targeted therapeutic interventions aimed at modulating aquaporin function to mitigate aberrant cellular responses associated with mechanical stress, thereby offering promising avenues for the treatment of various diseases and conditions characterised by dysregulated fluid homeostasis.

3.5 F-actin remodelling and actin crosslinkers in EdC cell volume regulation

Actomyosin networks and active contractility can orchestrate the intricate process of morphogenesis, ultimately contributing to tissue shaping and organ formation (Bailles et al., 2022, Vignes et al., 2022, Collinet and Lecuit, 2021, Munjal and Lecuit, 2014, Lecuit et al., 2011, Munjal et al., 2021). In the context of zebrafish cardiac development, the dynamic regulation of AVC EdC volume decrease during early valve formation coincides with F-actin remodelling and active cell contractility, specifically in the cells responsible for forming the AV valve (Vignes et al., 2022). Intriguingly, in *aqp8a.1* mutant embryos, which exhibit increased AVC EdC volume and ensuing AV valve defects, I found that F-actin remodelling is absent in EdCs located at the AV valve-forming region, indicating a correlation between the loss of Aqp8a.1 and AVC F-actin remodelling.

Additionally, actin crosslinking proteins are pivotal for the geometrical architecture of F-actin and, consequently, contractility. Their significance has been highlighted in numerous studies, where they have been shown to connect filaments, forming networks with various structures, geometrical arrangements, and contractile properties (Laporte et al., 2012, Koenderink and Paluch, 2018, Reymann et al., 2012, Chugh et al., 2017, Pollard and Wu, 2010, Svitkina and Borisy, 1999). As a result, these proteins play a crucial role in morphogenesis, contributing to the dynamic organisation of the actin cytoskeleton and spatiotemporal regulation of actomyosin contraction (Krueger et al., 2019). While a critical interplay between actin dynamics and AV valve development is implied (Vignes et al., 2022), the necessity of F-actin remodelling and the implication of actin crosslinkers in EdC volume modulation had not been investigated.

By analysing RNA sequencing data from the Vermot lab, I identified that *marcks1a* and *marcks1b*, paralogues of Marcks1 encoding for an actin crosslinking protein (Björkblom et al., 2012, Wohnsland et al., 2000), are expressed in the embryonic heart during early stages of valvulogenesis. Interestingly, previous research has linked the expression of Marcks1 to the regulation of endothelial cell size and blood vessel diameter (Kondrychyn et al., 2020). Hence, I decided to assess the potential role of Marcks1 in F-actin remodelling and its impact on AVC EdC volume regulation and valvulogenesis by studying *marcks1a* and *marcks1b* double zebrafish mutants.

My analysis revealed a significant impairment in F-actin remodelling in the absence of Marcks1, with no discernible F-actin enrichment observed in the AVC EdCs. These data suggest that Marcks1 is essential for the organisation of F-actin during AV valve formation. Strikingly, I demonstrated a significant increase in AVC EdC volume in *marcks1a* and *marcks1b* mutants, indicating that Marcks1 and, thereby,

F-actin remodelling are required for AVC EdC volume modulation. Moreover, I found that the loss of Marcksl1 does not affect the formation of the valve. This observation could be explained by potential adaptation-derived genetic compensation mechanisms involving other actin crosslinking proteins that may be expressed in the mutant conditions (El-Brolosy et al., 2019, El-Brolosy and Stainier, 2017). In future studies, generating RNA sequencing data from *marcksl1a* and *marcksl1b* mutants will be crucial in enhancing our understanding of these complex mechanisms.

3.6 The current model

Taking everything into account, the current model proposes that: Mechanical forces generated by blood flow are sensed by Piezo ion channels, triggering Ca^{2+} influx. Incoming Ca^{2+} tightly modulates the expression levels of *notch1b*, which subsequently controls *aqp8a.1* expression. At the protein level, CaM regulates the localisation of Aqp8a.1, by influencing its abundance in the cell membrane. Ultimately, Aqp8a.1 mediates water efflux and cell volume decrease. In parallel with these events, F-actin remodelling is essential for AVC EdC volume modulation (**Figure 52**).

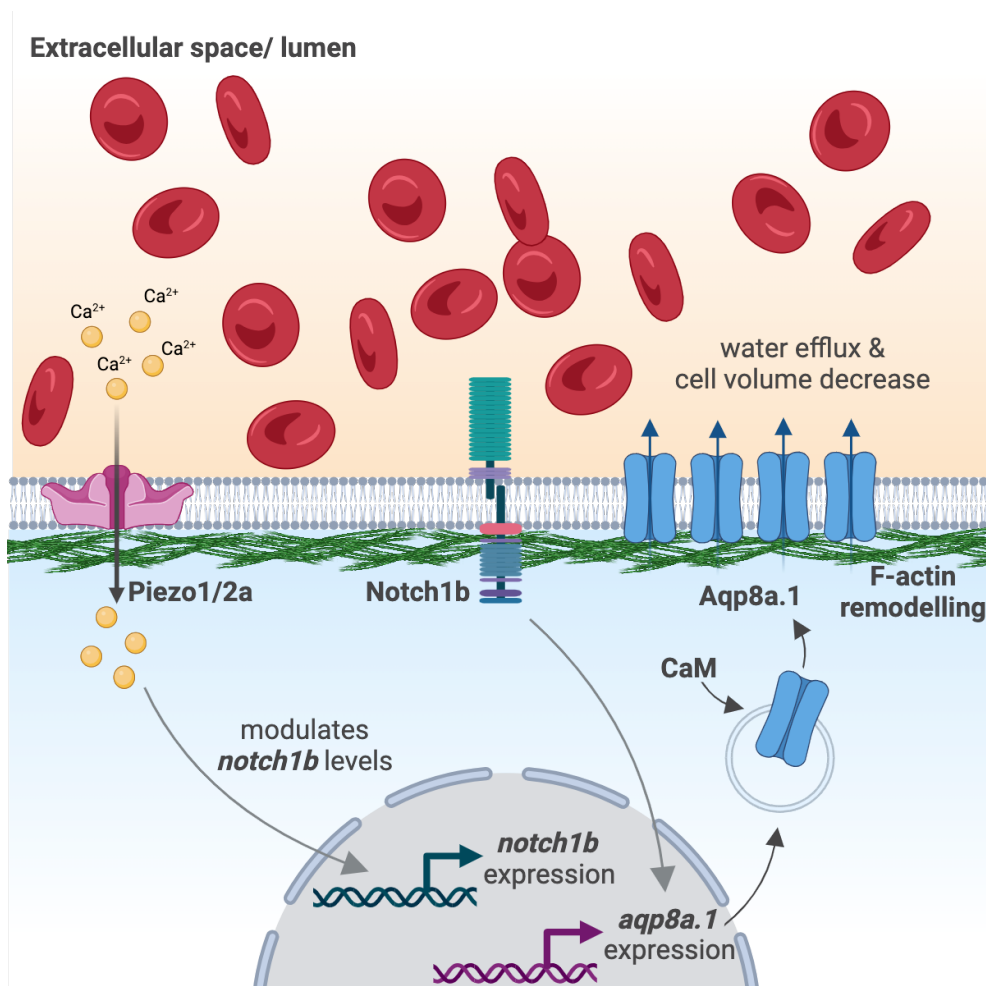


Figure 52 | AVC EdC volume regulation mechanisms. Schematic illustrating the current model for AVC EdC volume regulation during early AV valve morphogenesis. (Created with Biorender.com).

Chapter 4. Materials and Methods

4.1 Zebrafish husbandry

Animal experiments conducted in the UK adhered to the European directive 2010/63/EU and Home Office guidelines under the project licence PP6020928. Animal experiments conducted in Japan were approved by the Institutional Animal Care and Use Committee at RIKEN Kobe Branch (IACUC). Embryos were raised at 28.5 °C and treated with 0.003% 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich, P7629) after 50% epiboly to prevent pigment formation. We used the AB strain as wild type. The following published zebrafish lines were used in this study: *aqp1a.1^{rk28}*, *aqp8a.1^{rk29}* (unpublished- Phng lab), *piezo1^{bns340}*, *piezo2a^{bns367}*, (Juan et al., 2023), *marcksl1a^{rk23};marcksl1b^{rk24}* (Kondrychyn et al., 2020), *Tg(hsp70l:GenEPi)* (Yaganoglu et al., 2023), *Tg(kdrl:nls-mCherry)^{is4}* (Wang et al., 2010), *Tg(fli1:Has.B4GALT1-mCherry)^{bns9}; (kdrl:NLS-EGFP)^{ubs1}* (Kwon et al., 2016), *Tg(kdrl:mCherry-CAAX)* or *Tg(kdrl:Has.HRAS-mCherry)^{s916}* (Hogan et al., 2009). Newly generated transgenic lines included: *Tg(fli1ep:aqp8a.1-mEmerald)*, *Tg(fli1ep:aqp8a.1-mCherry)*, *Tg(fli1ep:aqp1a.1-mEmerald)*, *Tg(fli1ep:aqp8a.1^{Δ234}-mEmerald)*, *Tg(fli1ep:aqp1a.1^{Indel}-mEmerald)*. The primers listed in **Appendix A.1** were used for genotyping the mutant and wild-type alleles described in this study via PCR. PCR amplifications were performed with KAPA2G Fast Ready Mix (Sigma-Aldrich, KK5101).

4.2 Mutagenesis

Site-directed mutagenesis was performed using the Q5 Site-Directed Mutagenesis Kit (NEB, E0554S) according to the manufacturer's guidelines. Primers for site-directed mutagenesis were designed using NEBaseChanger

(<https://nebasechanger.neb.com/>) and are provided in **Appendix A.1**. Sequencing of clones was conducted by Eurofins Genomics (<https://eurofinsgenomics.eu/>). Plasmid DNA extraction was performed using the Qiagen Plasmid Midi Kit (Qiagen, 12143).

4.3 Confocal imaging

For fluorescent imaging of the beating or stopped heart, dechorionated zebrafish embryos were treated with either 0.16 mg/mL tricaine (Sigma-Aldrich, A5040) to inhibit fish motion or 50 mM 2,3-butanedione monoxime (BDM) (Sigma-Aldrich, B0753) to inhibit heart contraction. Anaesthetised embryos were mounted on a glass-bottom Petri dish (MatTek, P35G-0-14-C) in 1% low-melting point agarose (Invitrogen, 16520050) in E3 medium containing 0.003% PTU and supplemented with the same concentration of tricaine or BDM as the media.

Confocal imaging was performed on a Leica SP8 confocal laser scanning microscope equipped with a white light laser (WLL, Leica) using an HC PL APO CS2 20x/0.75 dry objective or on a STELLARIS 8 confocal laser scanning microscope equipped with a white light laser (WLL, Leica) using an HC PL APO CS2 20x/0.75 dry objective. Additionally, a Leica DMI8 combined with a CSU-X1 (Yokogawa, Tokyo, Japan) spinning at 10,000 rpm, 2 simultaneous cameras (TuCam Flash4.0, Hamamatsu, Shizuoka, Japan), and a water immersion objective Leica 40x/1.1, as well as an inverted Olympus IX83/Yokogawa CSU-W1 spinning disc confocal microscope equipped with a Zyla 4.2 CMOS camera (Andor) and an Olympus UPLSAPO 30x/1.05 silicone oil immersion objective were used.

Where necessary, realignment of the beating heart was performed post-imaging using BeatSync2.0 (Liebling et al., 2005).

4.4 Immunofluorescence

Immunofluorescence experiments were conducted on deyolked larvae. Zebrafish embryos were fixed at the desired stage for 2-3 h at room temperature (RT) or overnight at 4 °C in 4% w/v paraformaldehyde (Alfa Aesar, A11313) in PBS supplemented with 4% w/v sucrose (Alfa Aesar, J64270) and 120 µM CaCl₂ (Sigma-Aldrich, C1016) to facilitate deyolking. Following fixation, embryos were washed in PBS containing 0.1% Tween20 (PBST). For phalloidin (Alexa Fluor 488, Life Technologies A12379, 1:400), anti-fli1, and anti-VECadherin staining, embryos were permeabilised with 1x PBS containing 0.5% Triton overnight at 4 °C and subsequently blocked in PBS containing 0.1% Triton X-100, 5%BSA (SEQENS IVD, 1005-70), and 10% NGS (Coger, VS-1000) overnight at 4 °C. Antibodies were used as follows: rabbit anti-fli1 (Abcam, ab133485) 1:100, rabbit anti-VECadherin (kind gift from Affolter lab, (Blum et al., 2008)) 1:500, goat anti-rabbit Alexa-647 secondary antibody (Life Technologies, A-21235) 1:500.

4.5 Image analysis

4.5.1 Classification of cell polarity

Manual labelling of individual nuclei and Golgi apparatus was performed using the “cell counter” tool in FiJi/ ImageJ (Schindelin et al., 2012). Cell polarity was classified visually based on the position of the Golgi apparatus relative to the cell nucleus: polarised towards the outflow, polarised towards the inflow, or no clear polarisation.

4.5.2 F-actin intensity measurements

For immunofluorescent images with phalloidin labelling, we analysed 10 cells located at the AVC region of each sample. In each cell, 10 lines were drawn perpendicular to the plasma membrane using FiJi/ ImageJ (Schindelin et al., 2012), and the maximum intensity was measured and used for statistical analysis. A total of 7-10 embryos were used across different studies.

4.5.3 Nuclei segmentation

Nuclei segmentation was automatically obtained through the deep learning model StarDist3D, trained on N fish, incorporating both fixed and stopped hearts. The training of this supervised method needs ground truth labels, which were created using an internally developed simple tool for efficient semi-automatic blob instance segmentation. This method relies on simple image processing filters such as the Laplacian of Gaussian and user input to locally create 3D nuclei instances. This method allowed the creation of fully segmented 3D images in less than 30 minutes (depending on image complexity), and an iterative approach leveraging StarDist3D was used to generate and correct additional segmentations and increase the training dataset's size. The first 10 images were obtained through the interface, and the final dataset is composed of N images. Network training used patch size 96x96x96, batch size of 2, 100 steps per epoch for 500 epochs, 96 rays, and a UNet depth of 3. The remaining parameters are the default parameters of the public implementation (<https://github.com/stardist/stardist>). Classes were first assigned using the same simple interface, with manual selection of segmented nuclei instances, and then by leveraging the semantic segmentation (with properly labelled Atrium, AVC and Ventricle) of the endocardial lumen as a support for automated classification. In all cases, segmentations and classification are both examined by an expert agnostic of the biological hypothesis tested and corrected

accordingly. Nuclei volume and distances to the nearest neighbours were obtained through custom Python scripts.

4.6 *In situ* hybridisation

Whole-mount RNA *in situ* hybridisation (ISH) was conducted following established protocols (Thisse and Thisse, 2008). The *in situ* probes for *piezo1*, *piezo2a*, and *aqp8a.1* were synthesised as previously described (Juan et al., 2023, Vignes et al., 2022). The ISH samples were imaged using a Leica M165 C stereoscope fitted with Leica IC80 HD camera.

4.7 RNAscope

RNAscope experiments were performed on deyolked embryos fixed overnight at 4 °C in 4% w/v paraformaldehyde (Alfa Aesar, A11313) in PBS supplemented with 4% w/v sucrose (Alfa Aesar, J64270) and 120 µM CaCl₂ (Sigma-Aldrich, C1016) to facilitate deyolking. Whole-mount RNA *in situ* hybridisation was performed using the RNAscope Fluorescent Multiplex kit (Advanced Cell Diagnostics, 323110), according to the manufacturer's standard protocol. Embryos were imaged using an inverted Olympus IX83/Yokogawa CSU-W1 spinning disc confocal microscope equipped with a Zyla 4.2 CMOS camera (Andor) and an Olympus UPLSAPO 30x/1.05 silicone oil immersion objective. For quantification, the maximum intensity of the AVC RNAscope signal in the projection channel was measured and used for statistical analysis.

4.8 Pharmacological treatments

4.8.1 DAPT treatment

Zebrafish embryos were treated with 100 mM DAPT (Sigma-Aldrich, D5942) diluted in DMSO from 30 hpf to 48 hpf and kept at 28.5 °C.

4.8.2 Trifluoperazine dihydrochloride (TFP) treatment

Zebrafish embryos were treated with 100 µg/ml TFP (Sigma-Aldrich, T8516) diluted in dH₂O from 28 hpf to 48 hpf and kept at 28.5 °C.

4.9 Bead microsurgery

Microsurgery to insert a magnetic bead, approximately 30-40 µm in diameter, into the atrium of zebrafish hearts was conducted following previously established protocols (Fukui et al., 2021, Chow et al., 2022) at 54 hpf. In summary, embryos were anaesthetised with 0.16 mg/mL tricaine and mounted ventral side up in 1% low-melting point agarose dissolved in E3 medium supplemented with the same concentration of tricaine. Beads (Cube Biotech, 33201) were deposited on top of the solidified agarose, and a hole was created in the centre of the yolk using forceps. The bead was then gently pushed anteriorly until it reached the venous pole, where heart contractions caused suction, moving the bead into the cardiac lumen. Subsequently, embryos were released from the agarose and allowed to develop in E3 medium at 28.5 °C. Injected hearts in which the bead moved beyond the outflow tract or where the chambers collapsed were excluded from this study.

4.10 Heat-shock of zebrafish embryos

Piezo1 overexpression in *Tg(hsp70l:GenEPi)* was induced following a 1 h heat-shock at 37.5 °C. For animals used at 36 hpf, a heat-shock was conducted at 24 hpf, as the signal typically develops after approximately 4 hours (Yaganoglu et al., 2023). For animals used at 48 hpf, a subsequent heat-shock was performed at 36 hpf.

4.11 Statistical analysis

For statistical analysis of the acquired data, Student's paired t-tests with a two-tailed distribution or Mann-Whitney tests with a two-tailed distribution were conducted using Prism software. P values were represented as follows: < 0.05 *, < 0.01 **, < 0.001 ***, < 0.0001 ****.

The specific statistical test performed on the data, along with the number of analysed cells (n) and/or the number of embryos (N), and the interpretation of error bars, are provided in the figure legends. Plots were generated using Prism software, where the horizontal lines indicate the mean value and error bars show the Standard Error of the Mean (SEM).

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Appendix

A.1 List of primers

PCR genotyping	Forward primer (5'>3')	Reverse primer (5'>3')
<i>piezo1</i> ^{bns340} mutant specific	GAGCTTCGAGCTGTGATGGTT	TTCTGTTTCCCGGCTACACTTGATG
<i>piezo1</i> ^{bns340} wild-type specific	GCATCTACTTCACCTTGTGTCAGCG	TGTGGTGTCTGTCCAAACCCAG
<i>piezo2a</i> ^{bns367} mutant specific	TTCAGGTTCCGGTTGGTGCCGACG	TTCTCGGACTCTCTCCAGCACTTG
<i>piezo2a</i> ^{bns367} wild-type specific	TTCCGGTTGGTGCCGTTCTGAC	TTCTCGGACTCTCTCCAGCACTTG
<i>marcks1a</i> ^{rk23}	GTGTGTGTGTTTGCCAAGATGCATT	GGTTGCCACAGCGATATGAGATCAC
<i>marcks1b</i> ^{rk24}	AGCGCTGTAGGACTGGAAGTGGTA	CAACGACAGAAATGAATCGAAAACG

PCR mutagenesis	Forward primer (5'>3')	Reverse primer (5'>3')
<i>aqp8a.1</i> ^{Δ234}	TCAGGAGATCCACCGGTC	TCCAACCCAATAAATCCAGTG
<i>aqp1a.1</i> ^{Indel}	TTGGTAATGGGTGACAAGAAGGTCC GTGTTATTTTCAAAGGTGGCTCTGGA GGTGTG	TCTCACAATGCTGACAGTGACCAGAGC GCCGGTAAGAGGTCGCACACGCTCAG GGAAATC

ISH probes	Forward primer (5'>3')	Reverse primer (5'>3')
<i>aqp8a.1</i>	TAATACGACTCACTATAGCTGAAGC TCCGGGCAG	ATTTAGGTGACACTATAGCCTCTTCAGT TCCTTCTTC
<i>aqp1a.1</i>	GTCATGAACGAGCTGAAGAGC	GGGTCACTTTGAGGACATCTC
<i>piezo1</i>	CGCAATTAACCCTCACTAAAGGGTC TTCATCATCAAGTGTAGCCGGG	CGCTAATACGACTCACTATAGGGATCA TGGTCTCTGGAGAGCGATAG
<i>piezo2a</i>	CGCAATTAACCCTCACTAAAGGGCG CTCACATCTTTGTGCTCAAGTG	CGCTAATACGACTCACTATAGGGGAGC GGTACAGGAAGATCAGTTTG

A.2 The human PIEZO1, and the zebrafish Piezo1 and Piezo2a protein sequences considered in this study

>Human_PIEZO1

MEPHVLGAVLYWLLLPCALLAACLLRFSGLSLVYLLFLLLLPWFPGPTRCGLQGHGTGRLL
RALLGLSLLFLVAHLALQICLHIVPRLDQLLGPSCSRWETLSRHIGVTRLDLKDIPNAIRLV
APDLGILVSSVCLGICGRLARNTRQSPHPRELDDDERDVDASPTAGLQEAATLAPTRR
SRLAARFRVTAHWLLVAAGRVLAVTLLALAGIAHPSALSSVYLLLFLALCTWWACHFPIS
TRGFSRLCVAVGCFGAGHLICLYCYQMPLAQALLPPAGIWARVLGLKDFVGPTNCSSP
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DQWPQERESDQHVVPTAPDTEADNCIVHELTGQSSVLRRPVPRKRAEPREASPLHSLG
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TLCCLRYVWAMDRLPELPTTLGPVSLRQLGLEHTRYPCLDLGAMLLYTLTFWLLLROFV
KEKLLKWAESPAALTEVTVADTEPTRTQTLLQSLGELVKGVYAKYWIYVCAGMFIVVSFA
GRLVVYKIVYMFLFLLCLTLFQVYYSLWRKLLKAFWWLVAYTMLVLIAYVTFQFQDFPA
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EVSVMNLLLVLWAFALPYPRFRPMASCLSTVWTCVIIVCKMLYQLKVNPQEYSSNCT
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QEHYRRQHQLAPLPAQAVFASGTRQQLDQDLLGCLKYFINFFFYKFGLEICFLMAVNVI
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LLDRRLRIPELEEAELFAEGQGRALRLLRAVYQCVAAHSELLCYFIILNHMVTASAGSLV
LPVLVFLWAMLSIPRPSKRFWMTAIVFTEIAVVVKYLFQFGFFPWNSHVLRRYENKPYFP
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GPGVPAATTEDHIQVEARVGPTDGTPEPQVELRPDTRRISLRFRRRKKEGPARKGAAAI
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>Zebrafish_Piezo1 (NCBI Reference Sequence: XM_691263.8)

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RRRRANIATISKYCLFLVFMVYQYVLCVGIPPALCIDYPWRWRSTVTIHSALVKWMYLP
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EKNHN

>Zebrafish_Piezo2a (NCBI Reference Sequence: XM_021470255.1)

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LFEDFELRDGDCELPENEEEEEEAADADDGDLEEDIEESTKTRILRQIADAASKLKEIVG
NLITSAGKVWVTVLLGLTGMMPLSLTSSVYFFTFGLGLCTWWSFCRSFDPLLFSCLCVLMA
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