DISSERTATION

THE VOLTAGE GATED CALCIUM CHANNEL β2 PROTEIN IS REQUIRED IN THE HEART FOR CONTROL OF CELL PROLIFERATION AND HEART TUBE INTEGRITY

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2011

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ABSTRACT

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L-type calcium channels regulate calcium (LTCC) entry into cardiomyocytes. CACNB2 (β2) LTCC auxiliary subunits traffic the pore-forming CACNA subunit to the membrane and modulate channel kinetics. β2 is a Membrane Associated Guanylate Kinase (MAGUK) protein. A major role of MAGUK proteins is to scaffold cellular junctions and multi-protein complexes. To investigate developmental functions for β2.1, we depleted it in zebrafish using morpholinos. β2.1-depleted embryos developed cardiac edema and lethal cardiac defects. Ventricular cardiomyocytes proliferated at a slower rate, and failed to elongate their cell shape, which led to dysmorphic cardiac morphology and weakened contractility. Reduction in proliferation was marked by smaller heart fields and an increase in *bmp4*, an anti-proliferative marker. Thus, β2.1 helps regulate heart size by regulating the rate of mitosis and *bmp4* expression in the ventricle. Additionally, cardiomyocytes depleted for β2.1 failed to accumulate N-cadherin at the membrane, and

dissociated easily from neighboring myocytes under stress. Hence, we propose that β2 could function as a MAGUK scaffolding unit to maintain N-cadherin-based adherens junctions and heart tube integrity. To test this hypothesis we mutated the β2.1 residues necessary for interaction with the LTCC and observed its expression in cardiomyocytes using a GFP tag. Mutant β2.1 was still able to localize to the membrane supporting the possibility that it has a role in maintaining other protein complexes, such as adherens junctions.

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CHAPTER 1: INTRODUCTION

Cardiac development is a delicately timed process of gene expression and protein interaction. Changes in location, shape and function of cells determines heart morphology and its ability to function later in life. The L-type calcium channels (LTCC) link calcium entry to cardiomyocyte contraction, physically initiating the heartbeat. Of all the subunits comprising this channel, the β subunit determines how many LTCC are expressed at the membrane. Therefore it is an important determinant of optimal cardiac function, but whether it has additional roles outside of regulating channel kinetics and membrane localization is relatively unknown. The aim of this study is to further elucidate the role of the β subunit in cardiac development in zebrafish.

Zebrafish

The zebrafish, *Danio rerio*, is a freshwater species commonly found in India (Figure 1.1A). They belong to the Cyprinidae family which also includes carp and minnows. Their natural environment of shallow streams and stagnant pools of water makes them ideal model organisms for lab settings. Zebrafish can withstand being kept at high densities in relatively small volumes of water.

Originally, zebrafish research was pioneered by George Streisinger in the 1980's at the University of Oregon. Over the past 30 years the zebrafish has emerged as a powerful vertebrate model for many disciplines of biology. Currently zebrafish models

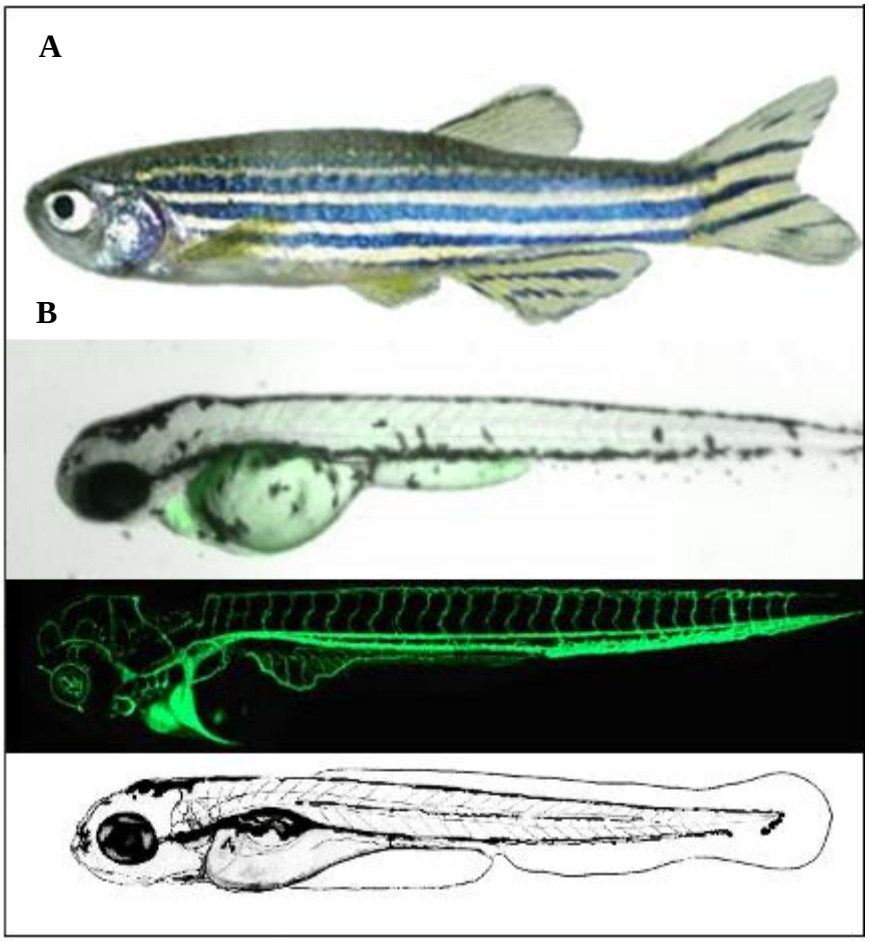

Figure 1.1: (A) Adult and (B) embryonic zebrafish at 4 days past fertilization. Zebrafish embryos commonly express transgenes that assist in tracking development of certain genes.

are employed for research in organogenesis, genetic models of human diseases, drug toxicity, circadian rhythm, regeneration and evolution (Yelon, 2001; Dooley et al., 2008; Brittijn et al., 2009; Ali et al., 2011; Ebarasi et al., 2011; Shao et al., 2011; Sukardi et al., 2011; Vatine et al., 2011).

Zebrafish undergo external fertilization, but sperm and eggs can be collected from fish individually and fertilized in vitro. One female can produce clutches of up to 200 embryos at a time. Its quick generation time and transparency make it ideal to study development; most organ primordia form within the first 36hrs of life, and some, like the heart, even earlier (Figure 1.1B). Zebrafish are especially geared towards cardiac development. Although the heart forms early, its function is not required till five days into development, since most of the embryo's oxygen needs are met by simple diffusion. Therefore experiments addressing heart function are not obscured by secondary side effect due to oxygen shortage.

Scientific Classification:

Kingdom: *Animalia*

Phylum: *Chordata*

Class: *Actinopterigii*

Infraclass: *Teleostei*

Order: *Cypriniformes*

Family: *Ciprinidae*

Genus: *Danio*

Species: *Rerio*

Heart Development

As an emerging model organism for heart development and function, the zebrafish shares many of its genes with humans, however some fundamental differences in the structure of the zebrafish heart must be addressed. Fish have gills instead of lungs; therefore their circulatory system is composed of only one loop, where the blood from the heart is pumped to the gills and the rest of the body (Figure 1.2A). In this scenario only two chambers are required to meet the body's oxygen needs. The human heart, in contrast, has four chambers and a two loop system in which the blood pumped to the lungs must first return to the heart before being sent to the rest of the body (Figure 1.2B). Septation of the mammalian heart does not extend to zebrafish and in that case a mouse model can be used. Regardless of differences in adult stages, early cardiac development progresses similarly in both these organisms, allowing the zebrafish to serve as a model for all vertebrate heart development.

Cardiac progenitors are already present in the blastula at 512 cells (Stainier et al., 1993) (Figure 1.3A). As gastrulation proceeds myocardial precursors arise in the lateral plate mesoderm (LPM), extending anteriorly in a patch located on either side of the embryonic midline (Figure 1.3B). These two primitive heart fields demonstrate elaborate patterning during early somitogenesis. The transcription factor, *nkx2.5*, is one of the earliest cardiac markers to be expressed. Although *nkx2.5* expression extends slightly beyond the area of myocardial precursors, it is necessary for the development of cardiomyocytes (Yelon et al., 1999).

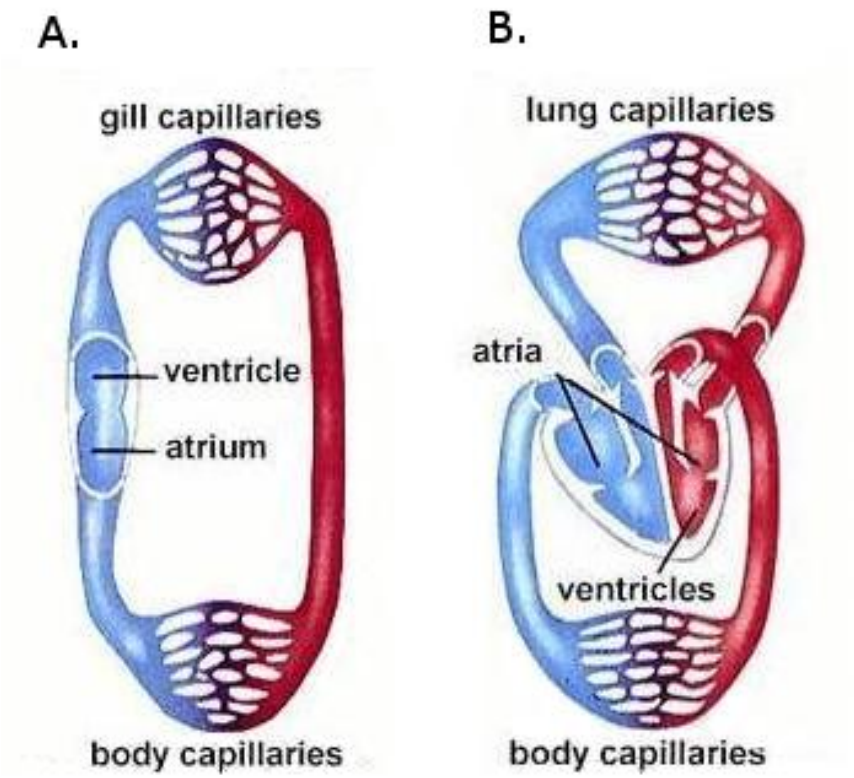

Figure 1.2: Diagrammatic depictions of a fish and a mammalian circulatory system.

(A) Fish hearts possess two chambers and a one loop circulatory system, (B) while mammalian hearts have four chambers and a two loop circulatory system. Adapted from http://gened.emc.maricopa.edu/bio/bio181/BIOBK/BioBookcircSYS.html#Vertebrate Vascular Systems

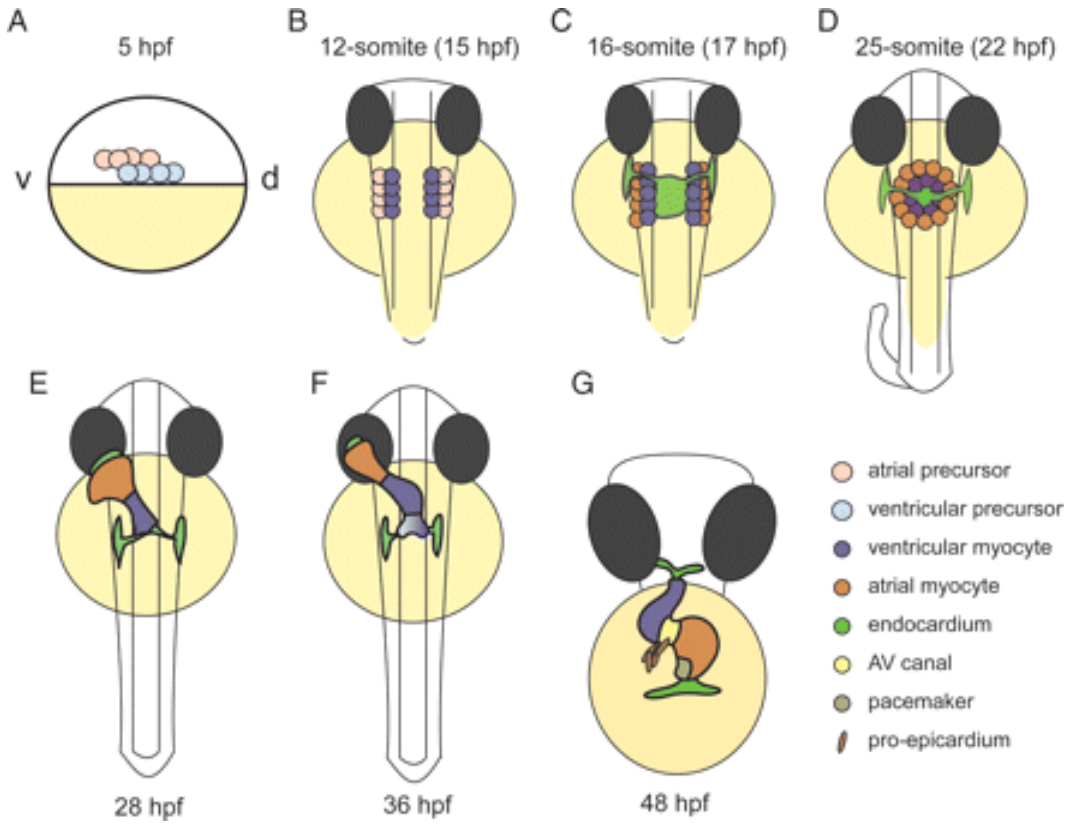

Figure 1.3: Cardiac development in zebrafish.

(A) Cardiac progenitor cells are located at the lateral regions of the embryo. (B) Following gastrulation the two populations of progenitors converge on either sides of the embryo. (C) Cardiomyocytes migrate towards each other with the assistance of signals from the endocardium. (D) The two cardiomyocyte populations converge and fuse into a heart cone. (E) The heart cone extends into a heart tube. (F) By 36 hours the atrium and ventricle are functional and morphologically distinct. (G) By 48 hours the heart tube undergoes looping and ballooning which places the two chambers next to one another and further expands their morphology (Stemmler, 2008).

Four genes have been identified as key regulators of *nkx2.5* expression: *gata5*, *fgf8*, *bmp2b*, and *oep* (Yelon, 2001). Mutating or reducing the expression of these early cardiac specification genes reduces the area of *nkx2.5* expression and leads to a smaller heart (Reiter et al., 1999; Marques et al., 2008). Likewise, exogenous expression of these factors leads to ectopic expression of *nkx2.5*, producing an expanded heart field (Reiter et al., 2001). Zebrafish express three *gata* genes in the LPM: *gata4*, *gata5* and *gata6*. All three of these transcription factors are capable of inducing some of the same myocardial genes; therefore it is likely that there is functional redundancy among the three *gata* genes (Charron and Nemer, 1999; Yelon, 2001).

Following specification cardiomyocyte precursors are aligned in two sheets on either side of the embryonic midline (Figure 1.3B). The next step to forming a functional heart is cardiomyocyte migration to the midline, followed by fusion. During this process the two cardiomyocyte populations migrate medially, towards each other, and fuse at the edges (Figure 1.3C). This fusion results in a cone and establishes the general position of different cardiac cells relative to one another; the atrial cells lie at the outermost perimeter, surrounding the ventricular cells. As it forms, the newly formed cardiac tube encloses the endocardial cells that will line the inside of the heart (Yelon, 2001). Although it is still unclear which signal initiates the cardiomyocyte movement, strong evidence suggests that endodermal cells are necessary, though not sufficient on their own, for cardiac fusion. Eliminating endodermal precursors leads to cardiac bifida, the formation of two individually independent hearts on either sides of the embryo (Alexander et al., 1999; Reiter et al., 1999; Reiter et al., 2001).

Once formed, the heart cone begins to tilt to the right and extend into a heart tube (Figure 1.3D, E). As soon as it is formed the heart tube begins to contract, but assembly of the contractile complexes probably begin earlier since mRNA expression of genes that will contribute to the sarcomeres precedes a functioning heart (Yelon, 2001; Smith et al., 2008; Bakkers, 2011). The initial contractions of the linear heart tube at 24hpf are peristaltic, reminiscent of a suction pump. Between 24-48hpf morphological changes, described below, give the chambers their characteristic kidney shape and looped chamber placement.

Chamber ballooning and looping transform the heart from a linear, tubular suction pump into a complex synchronized organ (Forouhar et al., 2006) (Figure 1.3F-G). This transition is a delicate balance between hemodynamic forces and cardiac function which translate into intracellular messages. The final shape of the heart is dependent on directional growth of individual cardiomyocytes and regionally specific cardiomyocte proliferation. Each chamber of the heart is subdivided into two regions; one that will become the outer curvature (OC) and one that will remain the inner curvature (IC) (Auman et al., 2007). The cells in the OC increase in surface area faster than the cells in the IC. The increase in size is also accompanied by an increase in length, so that the longest dimension of the cells lies perpendicular to the flow of blood (Auman et al., 2007). The cells of the OC are also more mitotically active. The increase in number and area of individual cardiomyocytes allows the OC to expand disproportionately to the IC, hence giving the chamber a kidney shape.

Mechanisms Controlling Cardiac Size

A fully functioning heart must develop to a certain size in order to carry out effective circulation. Therefore mechanisms that regulate cardiac growth are relevant to cardiac function and development. The analysis of mutant phenotypes suggests that heart size can be regulated at three different levels: specification of cardiac precursors, rate of proliferation or apoptosis, and the hypertrophy of individual cardiomyocytes.

Although the exact timing of cardiomyocyte specification is poorly understood, it must happen early on since cardiac lineage tracing studies indicate that precursors for both chambers already exist prior to the onset of gastrulation at 40% epiboly (Keegan et al., 2004). Multiple genes expressed throughout heart development may determine which cells take on the cardiac lineage (Vong et al., 2006; Zhou et al., 2008a; Langenbacher et al., 2011). However, several early factors need to be mentioned in detail since they are some of the most upstream signals in specification. Their expression is maintained in the heart throughout development yet their function may change depending on time and cardiac region of expression (Marques et al., 2008; Marques and Yelon, 2009). For example, *tbx2* regulates cardiomyocyte proliferation in the ventricle, but cell size in the atrium (Sedletcaia and Evans, 2011).

Bone mophogenic protein (BMP) signaling in cardiac specification and induction has been documented across multiple species (Shi et al., 2000; Nakajima et al., 2002). BMPs belong to the TGF-β family of signaling cytokines. Proteins in this family are known to regulate differentiation and proliferation. In addition to having cardiac

functions BMPs are vital for patterning the early embryo, particularly the ventral tissues (Wang et al., 2011). In early cardiac patterning BMP2 activates Nkx2.5, one of the earliest cardiac markers. Ectopic expression of BMP2 in mice is enough to induce Nkx2.5 expression and over-expression of BMP is associated with more cardiac progenitors being specified, but subsequently decreased proliferation of cardiomyocytes in mice (Prall et al., 2007). Eliminating BMP2 signaling in murine embryos is enough to completely stop heart formation whereas expanded levels of expression prior to heart tube formation lead to more atrial cardiomyocytes (Shi et al., 2000; Marques and Yelon, 2009). In the zebrafish heart BMP4 is uniformly expressed throughout the heart tube until after the start of development of cardiac cushions at ~36hp (Beis et al., 2005). At that time BMP4 expression becomes restricted to that area of the myocardium that lies directly adjacent to the future AVJ. Genetic evidence suggests that BMP4 signaling from the myocardium regulates the development of the AVJ, since inhibiting BMP4 activity in mice leads to valve and septation defects (Jiao et al., 2003).

Fgf8 is another important determinant of cardiomyocyte specification. It is expressed in the heart fields prior to cardiac fusion. Eliminating *fgf8* expression at different time stages in cardiac development generates different cardiac abnormalities (Marques et al., 2008). If *fgf8* is depleted in zebrafish during cardiac specification, the resulting heart will bear fewer cardiomyocytes in both chambers; conversely, reducing the amount of *fgf* after specification limits the cardiomyocyte reduction only to the ventricle (Marques et al., 2008). Interestingly, over-expression of *fgf* will only increase cardiomyocyte number if it occurs prior to specification.

Mechanisms for regulating the development and size of atria and ventricles are not the same. Evidence in support of chamber-specific developmental differences include observed differences in cell shape and gene expression; the atrial cells are squamous while the ventricular cells exhibit a more cuboidal shape (or fusiform, depending on stage of development) (Rohr 2008). The myosin genes expressed in the heart are also chamber specific, with the *atrial myosin heavy chain* (*amhc*) restricted to the atrium and *ventricular myosin heavy chain* (*vmhc*) expressed only in the ventricle (Yelon, 2001; Marques and Yelon, 2009).

The developing heart tube continues to be mitotically active even after it begins to function at 24hpf. Although the number of dividing cardiomyocytes is small, cell division still significantly impacts the overall population of cells in the heart. Factors which determine when and where cardiomyocytes should enter mitosis are important in determining the overall shape and size of the heart. Several genes are implicated in controlling the rate of proliferation in the embryonic heart: *isl-1*, *hand1*, *ndrg4* and *tbx2* (Risebro et al., 2006; Hayashi and Inoue, 2007; Qu et al., 2008; Sedletcaia and Evans, 2011). Depletion of Ndrg4 or tbx2 in zebrafish led to smaller hearts due to a decrease in cardiomyocyte proliferation in the ventricle. Ndrg4 in particular is required for progression through the cell cycle, and cells that lack it showed a decrease in cyclin D1 expression, arresting in the G1 phase of mitosis (Schilling et al., 2009). Evidence suggests that Tbx2 positively regulates Ndrg4 since its depletion led to decreased levels of ndrg4 transcript in the heart and phenotypes similar to Ndrg4 morphant zebrafish (Sedletcaia and Evans, 2011).

In addition to timely cell divisions, regional shape changes also determine the morphology of the developing heart tube. In order for the cardiac chambers to acquire their characteristic kidney shape, the cardiomyocytes in each chamber must grow in a regionally specific manner. Those cardiomyocytes closest to the outer curvature (OC) of the looping heart elongate perpendicular to the direction of blood flow, while cells in the inner curvature retain their small cuboidal shape (Auman et al., 2007). *nppa* (*anf*) in particular has been linked to the hypertrophy of embryonic cardiomyocytes via an unknown mechanism, since (OC) cardiomyocytes that fail to undergo the cell shape transition also express less *nppa* (Auman et al., 2007; Sedletcaia and Evans, 2011). This may not be the only mechanism that governs cell shape, since polarized cell shape changes have also been linked to the expression of adhesion molecules at the cell surface (Takeichi, 2011).

Cardiomyocyte adhesion

Development of tissues depends on adhesion of cells to each other and to the extracellular matrix (ECM). Contractile cells, such as cardiomyocytes, are especially dependent on adhesion proteins since they must be able to maintain intracellular and ECM connections under pressure and mechanical stress. Adhesion junctions can also transduce signals to second messengers and other relays in the cell that determine development and differentiation (Bugorsky et al., 2007; Chopra et al., 2011). Emerging evidence suggests that LTCC can be integrated into the adhesion signaling networks and determine the formation of adhesion junctions (Ko et al., 2001; Wu et al., 2001).

Although several intracellular adhesion junctions exist in cardiomyocytes adherens junctions are responsible for bearing and transducing mechanical force generated by contractions. N-cadherin joins cardiomyocytes together in a homophilic, calcium dependent manner (Noorman et al., 2009). On the cytosolic side of the cell, multiple accessory proteins link N-cadherin to cortical and sarcomeric actin (Noorman et al., 2009). Thus, the contractile machinery of each cardiomyocyte becomes one small link in a continuous chain. Contractile force is also counteracted by desmosomes; however, these form no direct attachments to sarcomeres. Instead desmosomes are joined to intermediate filaments (Schmidt and Jager, 2005). Defects in either in these adhesion junctions are linked to lethal cardiac developmental defects in mammals and zebrafish (Bagatto et al., 2006; Krusche et al., 2011).

In addition to physical adhesion cardiomyocytes must also share an electrical link. Conduction between cardiomyocytes is achieved by gap junctions, which form size selective pores that allow ions and metabolites to pass between cells and propagate the electrical impulse (Noorman et al., 2009; Chi et al., 2010). Desmosomes are composed of 12 connexins (6 of which are donated by each cell). In adult mammalian cardiomyocytes they localize to the intercalated discs and alternate with adherens junctions (Hoyt et al., 1989). However, in embryonic and neonatal cardiomyocytes, both junctions are evenly distributed across the plasma membrane at the onset of development and later re-localize to the intercalated discs (Noorman et al., 2009). A possibility exists that the localization of gap junctions is linked to that of adherens junctions, therefore defects in one may extend to the other (Matsushita et al., 1999).

Contracting sarcomeres also transduce tensile force to the ECM. Adjacent to each sarcomeric I-band is a costamere that links the contractile machinery to the cytoplasmic portion of integrins (Samarel, 2005). Integrins, in turn, attach and interact with the ECM, relaying extracellular signals to intercellular networks (Samarel, 2005). In vascular smooth muscle LTCC are located in close proximity to integrins. Signaling from the integrin receptor activates the LTCC and initiates an inward calcium current (Wu et al., 2001).

Genetic bases of human arrhythmias

Roughly one million people are diagnosed with cardiac arrhythmias each year (Priori et al., 2002). Many of these are inherited congenital heart defects resulting from improper ion channel function (Antzelevitch 2004). Some are linked to mutations in the LTCC or its auxiliary subunits. The first LTCC mutation to manifest as arrhythmia in humans was Timothy syndrome. This syndrome is caused by a mutation of the Cav1.2 gene, which encodes the pore-forming α subunit of the calcium channel. Arrhythmia is the most common symptom in those afflicted, but cardiac and developmental defects, paired with mental impairment also accompany this condition (Marks et al., 1995, Splawski et al., 2004). Timothy syndrome is associated with an autosomal dominant gain-of-function mutation. The cardiomyocyte cells become hyper-excitable because closing of the calcium channel is delayed. This prolonged action potential leads to prolonged inward calcium current which results in long QT syndrome, characterized by a longer QT interval on an electrocardiogram. On the other hand, Brugada syndrome, which also

leads to arrhythmias, is characterized by a short QT segment (Antzelevitch et al., 2007). Until recently, most cases of Brugada syndrome were linked to mutations in the sodium channel gene SCNA5 (Rossenbacker et al., 2004). In 2007, Antzelovitch et al identified novel mutations in the α and β subunits of the LTCC that both produced cardiac defects characteristic of Brugada syndrome. Thus, the LTCC is vital for cardiac function and development and further research is needed to elucidate its role in the cell and body.

L-TYPE CALCIUM CHANNELS

LTCC Function in the Heart

Calcium is an important second messenger in the cell that has widespread effects and interactions. Therefore its location and abundance must be tightly regulated in order to maintain continuity in gene expression and protein interaction. In an excitable cell in the resting state, the cytosolic Ca^{++} concentration is low. Calcium is sequestered within intracellular compartments or in the extracellular space. The L-type calcium channel is one way the cell regulates entry of calcium into the cytosol. Four members of the L-type calcium channel (Cav1.1-4) regulate muscle contraction, pace-making activity, and excitation-transcription coupling in neuronal and retinal function.

L-type calcium channels are embedded into the membrane of the cell (Figure 1.4). Their presence has been documented in smooth and striated muscle, neuronal tissue, kidneys, ear, and immune cells (Buraei and Yang, 2010). In the heart they contribute to

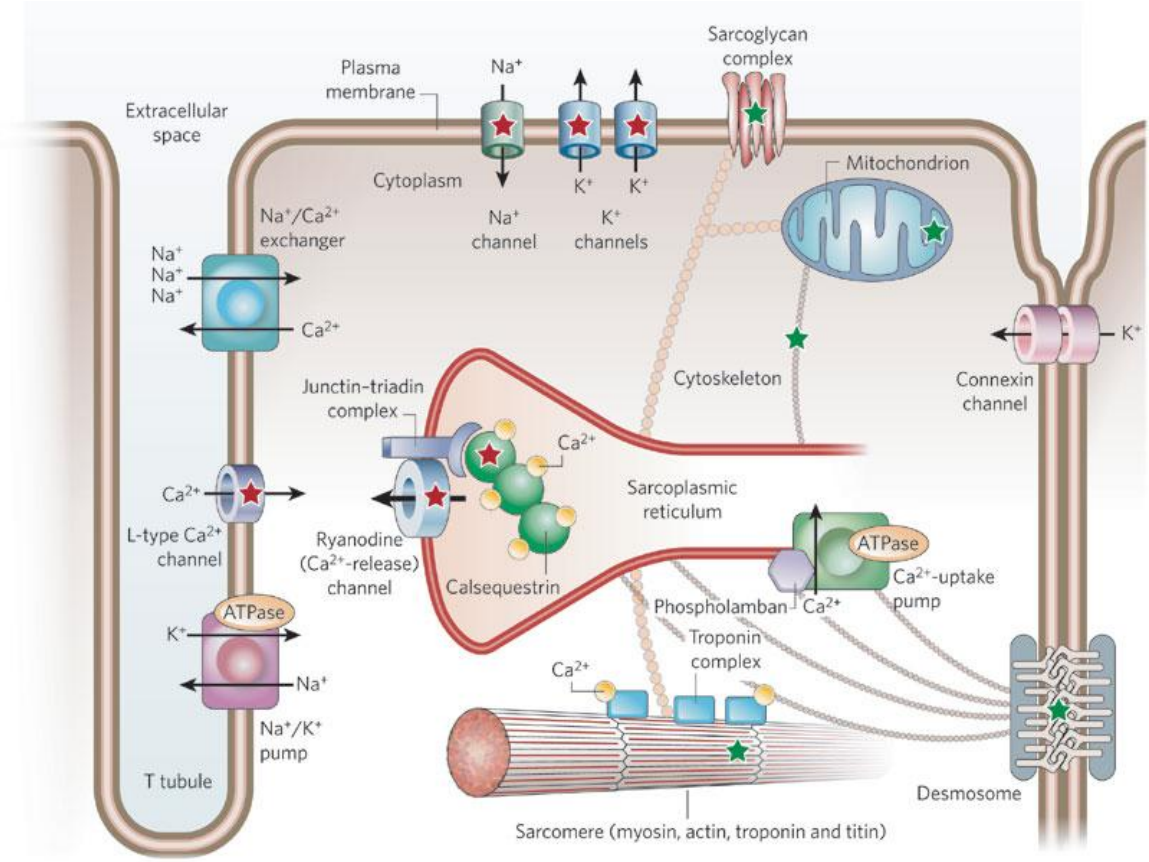

Figure 1.4: Depiction of calcium cycling and excitation-contraction coupling in the cardiomyocyte.

Ca2+ entering through the L-type calcium channel stimulates the Ryanodine Receptor (RyR) to release more Ca2+ from intracellular stores. The Ca2+ then binds and displaces troponin, and allows muscle contraction to occur. From (Knollmann and Roden, 2008).

the cardiac action potential by mediating calcium entry into the cell, and triggering calcium induced calcium release (CICR) from the sarcomplasmic reticulum (SR) (Fabiato 1983). The mechanism controlling CIRC varies slightly between cardiac and skeletal muscle. In cardiomyocytes LTCCs are in close proximity to the ryanodine receptors (RyR) located on the SR membrane, although they are not physically coupled (Figure 1.4). The calcium entering through the channel binds to the RyR and causes its opening. Skeletal muscle differs in that there is a direct protein-protein interaction between the voltage sensor of the LTCC and the RyR (Dulhunty et al., 2002). The resulting high concentrations of intracellular calcium facilitate the next systolic contraction (Bers 2002). This occurs via calcium binding to troponin C which shifts its location on the actin fiber allowing, for myosin interaction (Figure 1.4).

Structure and Function

The oligomeric L-type calcium channel is composed of four subunits: α1, γ, $\alpha_2\delta$, and β (Figure 1.5). The transmembrane α1 pore-forming subunit contains four homologous repeats of six transmembrane segments (Figure 1.6). Segment number four houses the voltage sensor which responds to membrane depolarization by opening the channel and allowing Ca^{2+} entry into the cell.

The membrane associated $\alpha_2\delta$ regulatory subunit has differential effects on calcium current depending on the $\alpha_2\delta$ isoform and the α1 subtype (Tuluc et al., 2007). Heterologous expression of $\alpha_2\delta$ in *Xenopus* oocytes suggests that it may have an effect on the propensity of the α subunit to associate with the plasma membrane

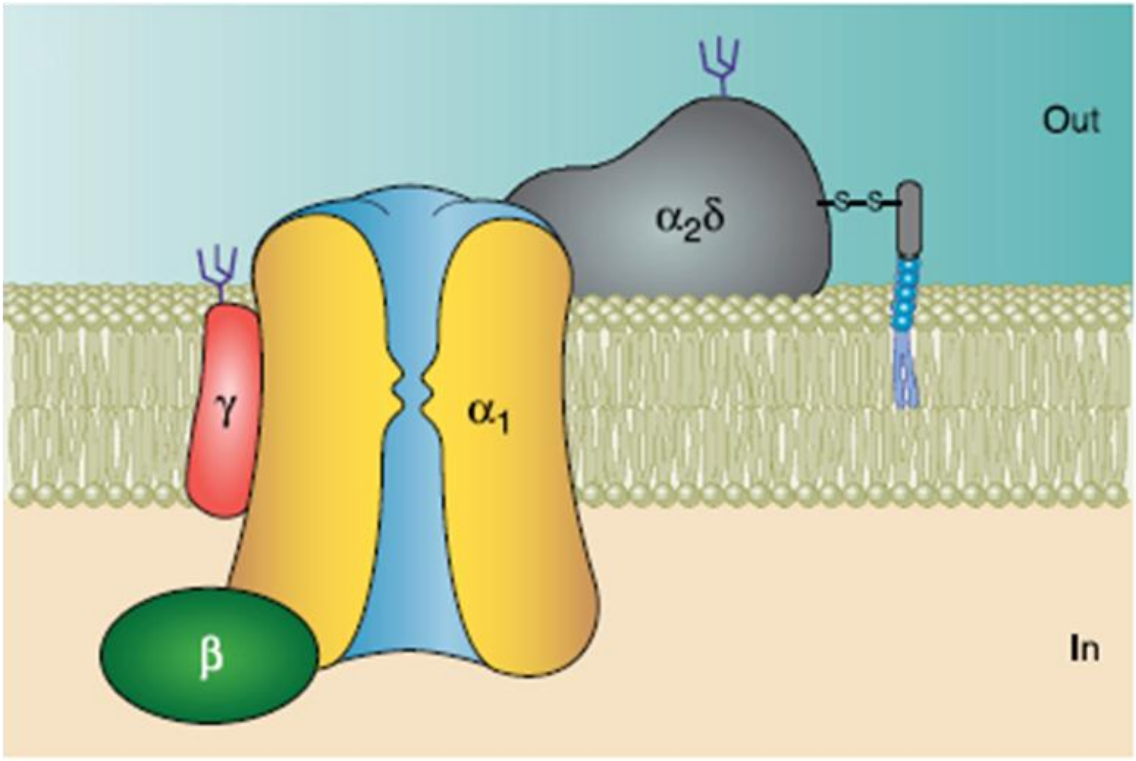

Figure 1.5: Subunit composition of the high-voltage activated L-Type calcium channel.

The α1 subunit composes the Ca2+ conducting pore of the channel. The three remaining subunits: β, α2δ and γ serve regulatory functions. Adapted from (Buraei and Yang, 2010).

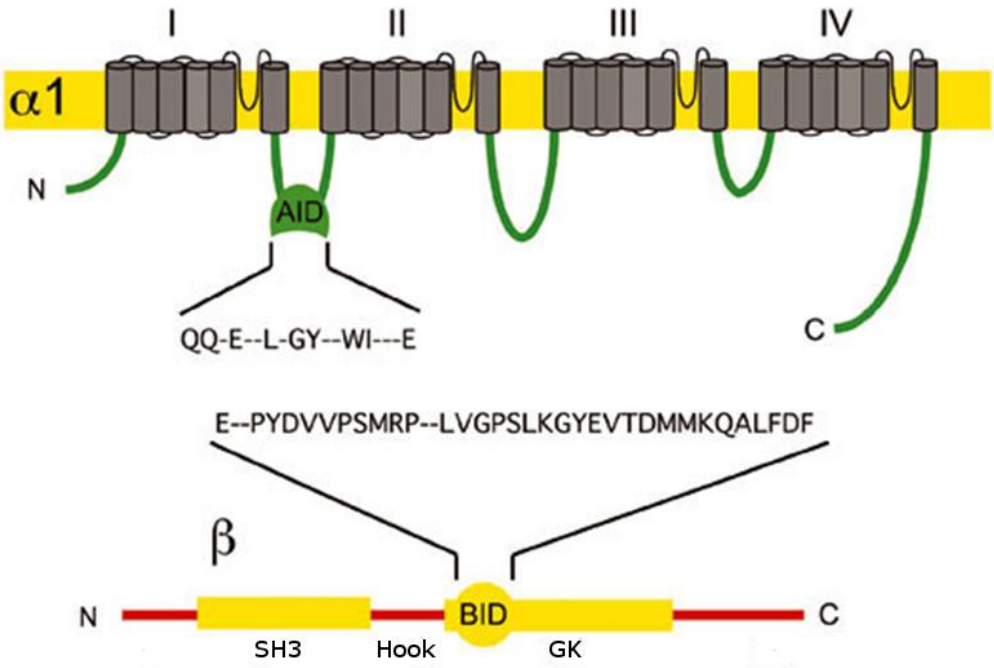

Figure 1.6: The conserved residues of the alpha interaction domain (AID) of the Ca^{2+} channel α1 subunit and the beta interaction domain (BID) of the β subunit are critical for α1/β binding and Ca2+ current expression. The conserved SH3 and GK domains are indicated in yellow on the β subunit. Adapted from (Wolf et al., 2003).

(Shistik et al., 1995). This subunit also contains an extracellular domain and could be involved in extracellular signaling (Figure 1.5) (Barclay et al., 2001).

The transmembrane γ subunit was thought to only exist in the skeletal muscle LTCC, but recently its presence has been detected in neuronal and cardiac tissue (Black, 2003; Yang et al., 2011). Its role in modulating the calcium channel is unclear, since γ subunit null mice are morphologically indistinguishable from wildtype and have no problems assembling the channel complex (Arikhath et al., 2003). The calcium current in $\gamma_1^{-/-}$ null mice is slightly increased (Freise et al., 2000). In vivo association assays suggest that the mutant phenotypes in mice deficient in the γ_2 subunit are not due to a LTCC abnormality but rather to additional biological roles for the γ's as chaperones of the AMPA receptors (Osten and Stern-Bach, 2006; Chen et al., 2007).

The membrane associated β subunit is responsible for most regulation of channel expression and gating (Caterall 2000). Decreasing the expression of β subunits in the cell greatly reduces the calcium current and α1 expression at the membrane (Vendel et al., 2006). In contrast, over-expressing β proteins increases the density of the endogenous calcium currents (Colecraft et al., 2002).

Electrophysiological Properties

Voltage-gated L-type calcium channels are distinguished from other channels by their sensitivity to pharmacological agents and membrane depolarization. Dihydropyridines (DHP) inhibit the activity of L-type calcium channels by binding to the α1 subunit but have no effect on other closely related calcium channels. Hence,

sometimes the LTCC is referred to as a dihydropyridine receptor. The L-type calcium channel is characterized by its sensitivity to high voltage and long open state. Typically, a depolarization of -30mV is enough to cause channel opening, although this number can vary depending on circumstances and cell type (Brette et al., 2006). The duration of channel opening and its sensitivity to depolarization are mainly regulated by the $\alpha_2\delta$ and β subunits.

β Subunit Structure and Function

The β subunit is the only completely cytosolic subunit of the calcium channel. It interacts with the α1 subunit at the alpha interacting domain (AID) (Figure 1.6A). Mammals express four β subunits (β1-β4) that interact non-exclusively with the α subunit in various voltage gated calcium channels. The expression of β subunits in the embryo and in the adult is temporally and spatially segregated (with some overlap) (Acosta et al., 2004 Schjött et al., 2003). β subunits contain five distinct domains: the N-terminus, Src-homology 3 (SH3) domain, HOOK, guanylate kinase (GK) domain and the C-terminus (Figure 1.6). Alternative spicing exists among exons encoding the N-terminus and HOOK regions, which accounts for all of the β subunit splice variants. In mammals, each of the four β proteins is associated with unique patterns of channel expression and electrophysical properties, yet their overall similar protein structure classifies them into the Membrane Associated Guanylate Kinsase (MAGUK) family of proteins (Buraei and Yang 2010). β subunit mRNA and protein are found in all excitable tissues as well as

some non-excitable tissues including kidney and liver (Ebert et al., 2008, Link et a., 2009, Pitchler et al., 1997). The wide distribution of β subunits throughout the body is consistent with their important roles in maintaining physiological functions.

The β's, like other MAGUKs, contain an SH3 domain as well as a catalytically inactive guanylate kinase (GK) domain, but lack the PDZ domain usually located at the N terminus and associated with protein interaction (Funke et al., 2005). The SH3 and GK domains are essential for β's ability to regulate channel gating. Mutating these domains or varying the flexibility of the HOOK region that separates them greatly reduces the β subunit's ability to affect channel gating (Chen et al., 2009). The AID domain of the α1 subunit interacts with residues known as the AID-binding pocket located within the GK domain of the β subunit (Figure 1.6). Mutating residues of the AID-binding pocket (ABP) can completely abolish α1/β interaction (He et al., 2007). Though it is well established that this is the main region at which the α1/β association occurs there are additional regions of the β N-terminus and HOOK domain that determine the properties of the β subunit. Swapping the HOOK domain of β1b with β2a switches the properties of voltage dependent inactivation (VDI) to that of β1b whereas removing the N-terminus abolishes VDI completely (He et al., 2007; Jangsangthong et al., 2010). Whether these functions are dependent on direct association of the N-terminus and HOOK domain with the α1 subunit has yet to be determined. However, in order for them to occur the β must be tightly anchored to the α1 via its AID-binding pocket (ABP). Mutating the residues of the ABP abolishes the interaction of all other domains with the α1 (He et al., 2007).

Previous hypotheses suggested that α1 expression at the membrane was dependent on β occluding an ER retention signal found in the α1 protein. Transferring the

I-II loop of the α1 (containing the AID) onto a K^+ channel greatly reduced its membrane expression, but this could be rescued by co-expression of the β subunit (Bichet et al., 2000). However, a recent discovery unveiled that the β subunit competes with RFP2 ubiquitin ligase for α1 binding. In the absence of β the α1 undergoes ubiquitination and rapid degradation (Altier et al., 2011). Thus, the β protein functions not only as an ER occlusion factor but as a stabilizing protein for channel expression.

Aside from ensuring that the α subunits are appropriate expressed at the membrane, the β subunits also dictate how the channel responds to changes in membrane potential. Each β endows the channel with different activation/inactivation kinetics based on its variable N-terminus and HOOK domains (Olcese et al., 1994; Yasuda et al., 2004; Hidalgo et al., 2006; He et al., 2007).

Additional Protein Partners of β subunits

In addition to the α subunit of the LTCC the β interacts with various other proteins, some of which further regulate its ability to interact with the calcium channel and others that play a role in channel independent functions (Colecraft et al., 2002; Ebert et al., 2008a). As described below, the partners of β proteins include protein kinases, ryanodine receptors, actin and histone binding proteins, and some transcription factors.

β subunits are phosphorylated by multiple kinases. Protein kinase A (PKA) can phosphorylate all four variants of β subunits and leads to an up-regulation of Cav1.2 currents (Vallentin and Mochly-Rosen, 2007). However, its affect is β specific, with the largest increase in current observed with β1 association and the smallest with β2

association (Vallentin and Mochly-Rosen, 2007). Thus, regulation of Cav1.2 currents via PKA is probably not essential in the heart where β2 is predominantly expressed (Hullin et al., 1992; Luo et al., 2005; Skurk et al., 2005). Protein kinase B (PKB) can phosphorylate β2, which leads to an up-regulation of LTCCs at the membrane. In the absence of PKB LTCCs enter a degradation pathway (Catalucci et al., 2009). A current model suggests that phosphorylation by PKB masks the signal required to trigger alpha ubiquitination (Catalucci et al., 2009). The induction of PKB is dependent on increased PIP3 levels which recruit PKB to the membrane and allow it to phosphorylate β2 (Risebro et al., 2006; Hayashi and Inoue, 2007; Langenbacher et al., 2011). PKB induction is especially vital to cardiac function. PKB knockout mice exhibit cardiomyopathy and premature death (Catalucci et al., 2009). Although not yet confirmed as a direct protein partner of β, PKC does phosphorylate α at the I-II loop in Cav2.2 and Cav2.3. Phosphorylation by PKC enhances channel activity in the presence of β subunits (Foley and Mercola, 2004; Sedletcaia and Evans, 2011). On the other hand, phosphorylation of β2a by PKG inhibits channel activity in cultured cells (Wang et al., 2011). In addition, Ca2+/calmodulin dependent kinase II (CamKII) also increases channel activity by phosphorylating β1b, β2a and Cav1.2 (Marques and Yelon, 2009).

Some proteins that interact with the β subunits are necessary for other physiologic functions beyond the maintenance of calcium channels. In skeletal muscle LTCC are arranged in tetrads adjacent to the ryanodine receptor (RyR) (Keegan et al., 2004; McCulley et al., 2008). This grouped arrangement and physical linkage of LTCC to the RyRs is completely dependent on β1a, which is expressed exclusively in skeletal muscle (Shi et al., 2000; Nakajima et al., 2002; Jiao et al., 2003; Schneider et al., 2003; Prall et

al., 2007). β1a directly binds the RyR at its C-terminus (Nakajima et al., 2002; Prall et al., 2007; Wang et al., 2010). Removing β1a from this confirmation abolishes calcium current and excitation-contraction (EC) coupling (Shi et al., 2000; Schilling et al., 2009). Replacing β1a with other β subunits can restore calcium channel function but not EC coupling.

Recently, a large scaffolding protein, termed Ahnak (700 kDa), was determined to bind to β2 in cardiac and skeletal muscle in addition to other non-excitable cell types (Radice et al., 1997; Haase, 2007; Krusche et al., 2011). Ahnak can bind and bundle actin. Through its interaction with Ahnak the β subunit provides a link from the LTCC to the cytoskeleton. It is unclear what domain(s) of the β subunits bind Ahnak, but since multiple different β's (β1, β3, β2) can co-precipitate with Ahnak, binding probably occurs within the conserved MAGUK domains (Radice et al., 1997; Alvarez et al., 2010; Kresh and Chopra, 2011). Whether this interaction has any functional significance is still a mystery, since Ahnak knockout mice show no phenotype and are viable (Komuro et al., 2004), but these data further emphasize β's role as a MAGUK protein.

Until recently β subunits have been thought to function exclusively with high voltage-activated calcium channels; even if they interacted with other proteins it was always in conjunction with the calcium channel. New evidence challenges this view and suggests regulatory functions for β subunits in gene expression that are independent of their role as calcium channel regulators.

Truncated versions of β4 subunits exist that lack most of the GK and SH3 domain. Hence their interaction with the α1 subunit is minimal (Hibino et al., 2003).

Yeast two hybrid screens showed that β4c can directly bind heterchromatin protein 1 (HP1). HP1 is an essential protein involved in gene silencing. Using GFP-tagged versions of the truncated chicken β4c isoform, Hibino et al., showed that it can translocate to the nucleus in the presence of HP1 and bind the chromo shadow scaffolding domain of heterochromatin protein 1 (HP1) (Hibino et al., 2003).The binding of β4c to HP1 diminished its gene silencing functions and implicated β4c as a transcriptional regulator (Hibino et al., 2003).

In 2010, Zhang et al., showed that β3 can directly interact with a new splice variant of Pax6 (Zhang et al., 2010). Pax6 is a transcription factor necessary for the proper development of the eyes (Mathers and Jamrich, 2000; Georgala et al., 2011). The binding of β3 to Pax6 suppressed its in vitro transcriptional activity. Co-expressing *pax6* and β3 with voltage gated-calcium channels in *Xenopus* oocytes does not change the channel properties, suggesting that at least one β subunit may have a dual function as a transcriptional regulator and LTCC subunit. In addition, β4a interacts with the nuclear regulatory subunit of phosphatase 2A (PP2A) (Barbado et al., 2009). As a complex these two translocate to the nucleus and associate with nucleosomes where they regulate the dephosphorylation of histones (Barbado et al., 2009).Thus, although initially surprising, the nuclear localization of β proteins does appear to be functionally significant.

β subunit variants and distribution

Although each of the four β subunits has distinct areas of localization and temporal expression there is some overlap and possible functional redundancy among

them. This, in addition to the many splice variants possible for each subunit, emphasizes the complex nature of β function. Most of the β subunits are expressed within the nervous system to some degree (Buraei and Yang, 2010). β1 is the major β subunit found in association with α1S in skeletal muscle, while β3 and β4 seem to be evenly distributed through many of the non-excitable tissues (Buraei and Yang, 2010). All of the different β's are documented in the heart, but the β2 subunit is the predominant β gene expressed in the adult and embryonic murine and zebrafish heart (Ebert et al., 2008c; Zhou et al., 2008b; Link et al., 2009). In 2008 Zhou et al., describe novel zebrafish splice variants of β2. Although they detected the expression of those β2 transcripts in embryonic zebrafish brain using ISH, no signal was present in the heart (Zhou et al., 2008b). Several β2 splice variants exist in the zebrafish as well as mammals, adding to the potential complexity of this protein's function and patterns of expression (Hullin et al., 1992). Thus, a likely explanation for the absence of β2 in the heart as observed by Zhou et al., could be the limitation of the ISH probes to detect other β2 splice variants. Its role as regulator of the L-type calcium channel in the heart is well documented in mammals. The expression of β2 subunit in the embryonic heart of mice suggests it may also contribute to morphology or function of the embryonic heart (Serikov et al., 2002).

Loss of β2 expression led to vascular and cardiac deformities in mice (Weissgerber et al., 2006). These phenotypes were associated with embryonic lethality by E10.5. Hearts of $\beta_2^{-/-}$ null mutant mice exhibited compromised cardiac performance including decreased cardiac output and slower heartbeats. In addition, $\beta_2^{-/-}$ mice exhibited anomalies in looping of the developing heart tube. The complexity of the vasculature was reduced, most likely as a secondary consequence of decreased hemodynamic forces. In

this report, we employ the zebrafish model system to study β2 function in cardiac development. Since zebrafish embryos aged 6 days or fewer receive oxygen by diffusion rather than cardiac output, development of the cardiovascular system can be tracked independently of the effects of hypoxia.

MAGUKs

The conserved SH3 and GK domain of β subunits classify them into the MAGUK family (Figure 1.7). MAGUKs are scaffolding proteins that establish a link between transmembrane proteins and cytoskeletal elements at adhesion junctions, and serve as intermediates in signal transduction pathways (Caruana, 2002). Their role as linkers and adapters determines cell polarity and influences organogenesis in the developing embryo (Caruana, 2002). As scaffolding proteins, they assemble junctions that can separate the apical and basal membranes of the cell. These junctions can form barriers that prevent the movement of apical proteins onto the basal membrane and vice versa, thereby conferring cell polarity (Park et al., 2006). In addition to SH3 and GK, many MAGUKS also contain a PDZ domain. This domain is thought to assist MAGUKs in clustering membrane proteins together (Semba et al., 2000; Park et al., 2006). Although no PDZ domain exists in the β subunits they have no difficulty assisting channels to the plasma membrane, typical of MAGUK functions (Semba et al., 2000). The three most renowned MAGUKs; ZO-1, Dlg and PSD-95 play many roles at the cell membrane.

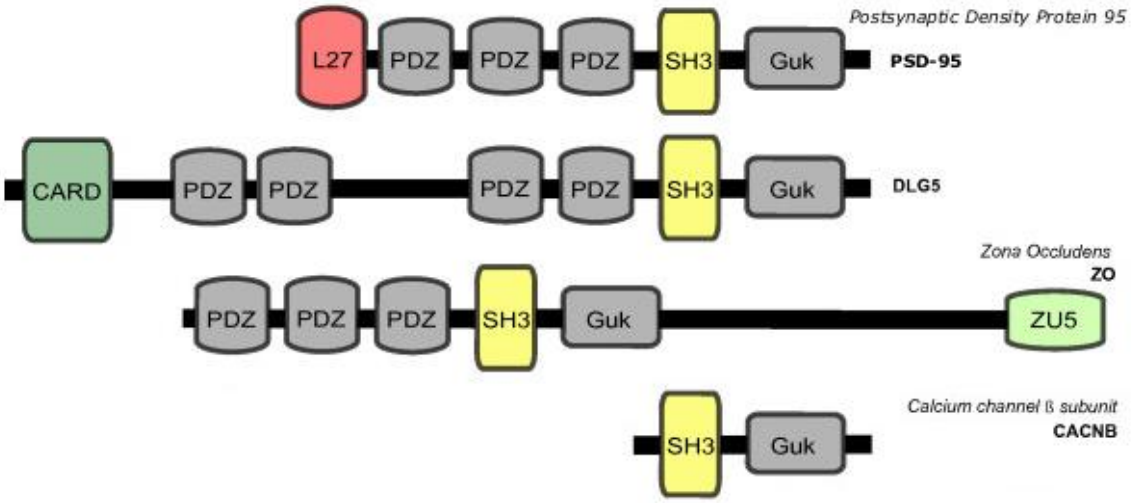

Figure 1.7: Comparison of several commonly studied MAGUK proteins in comparison to the β subunits.

All MAGUKS share the SH3 and GK domains, while the PDZ domain is absent from the β subunits. Adapted from (de Mendoza et al., 2010).

The zonula occludins (ZO) proteins, expressed at the membrane of epithelial cells, are essential for correct organization of tight junctions (Gonzalez-Mariscal et al., 2000). Different ZO proteins form complexes via their three PDZ domains and associate with complexes of junctional proteins, such as the transmembrane claudin and occludin (Gonzalez-Mariscal et al., 2000; Sadlon et al., 2004). ZO proteins also play a role in establishing cell polarity in mouse epithelial cells in conjunction with other adapter MAGUK proteins (Umeda et al., 2004). ZO function is not limited to the membrane. Recently ZO proteins have been shown to associate with G-proteins in MDCK epithelial cell lines and factors that regulate gene expression (Meyer et al., 2002; Bauer et al., 2010). It is likely that the β shares some of these functions as well since some β proteins translocate to the nucleus (Colecraft et al., 2002).

The Drosophila Discs Large (Dlg) MAGUK protein localizes at the cytoplasmic side of epithelial cell septate junctions (Papagiannouli and Mechler, 2010). In invertebrate septate junctions are analogous to the vertebrate tight junctions (Tepass and Hartenstein, 1994; Moyer and Jacobs, 2008). The homologous mammalian protein (also called Dlg) is also involved in the targeting of adherens junctions (Woods et al., 1996; Knollmann and Roden, 2008). It is classified as a tumor suppressor gene since mutating either the SH3 or GK domains of Dlg results in increased cell proliferation and failure to properly assemble septate junctions (Hough et al., 1997). This suggests that MAGUKs are not only important in regulating structural integrity of cell junctions but also the cell cycle.

Some MAGUKS function as synaptic scaffolding molecules. Formation and proper function of synaptic junctions depends on the MAGUK protein, PSD-95 (Han and

Kim, 2008). It interacts with adhesion proteins and junctional receptors at the post-synaptic membrane of neurons to influence synaptic plasticity (Funke et al., 2005).

Taken together, research shows that MAGUK proteins have membrane-related functions that determine many aspects of cell organization and organism development. Since the β subunit falls into the MAGUK family it may share some of these important roles.

Zebrafish β2 genes

The zebrafish genome encodes two β2 genes, *β2.1* and *β2.2*, consistent with the duplication of the zebrafish genome 450 million years ago (Jaillon et al., 2004; Ebert et al., 2008c). Of these, *β2.1* is the most similar to the human β2 (87 % amino acid homology compared to 62% for *β2.2*).

Aims of this study

This study aims to address several aspects of β2 function in the heart of embryonic zebrafish: 1) Since LTCC mutations lead to cardiovascular defects, we want to understand the linkage of β2 to human diseases. 2) Mice depleted of β2 protein die before birth from heart defects, thus exploring β2's role in cardiac development is also relevant to understanding vertebrate cardiogenesis. 3) We also explore potential LTCC or MAGUK roles for β2 in context of an animal model, since cultured cells may not adequately capture functions related to adhesion in development.

We report that depletion of β2.1 in zebrafish embryos results in reduced cardiac looping, decreased cardiac output, and weak contractility. The cardiac chambers are dysmorphic with dilated atria and collapsed ventricles, which may reflect defects in the assembly of contractile machinery (Auman et al., 2007). To explore the mechanistic basis for this phenotype, we investigated the specification of cardiac precursors within the bilateral heart fields, the rate of cell proliferation in the early heart tube, and the functional properties of the maturing cardiomyocytes. All of these properties were altered in β2.1- depleted embryos, indentifying the β2.1 protein as an essential component of embryonic heart development. Mutating the ABP of the β2.1 subunit did not significantly affect its membrane localization in the developing cardiomyocytes, suggesting the possibility of additional membrane associated roles, perhaps via its MAGUK domains.

CHAPTER 2: RESULTS

Depletion of β2.1 results in reduced cardiac function and defective morphology.

To determine the function of β2.1 in vivo we depleted its expression in zebrafish embryos using morpholino oligonucleotides. Since β2.1 is alternatively spliced at the 5' end, we designed the morpholino to target the splice donor site of exon 5, an internal exon that is common to all splice variants (Figure 2.1A)(Ebert et al., 2008c). Mis-splicing of the β2.1 transcripts is predicted to encode a frameshift which would truncate the protein at exon 6, resulting in a protein of 149 amino acids compared to the 579 residues of wildtype β2.1. If translated, the truncated protein would encode the SH3 domain; however, the conserved residues of the GK domain that are critically required for β subunit interaction with the α subunit would be missing. Hence, the truncated β2.1 subunit is not anticipated to interact with the calcium channel.

RT-PCR was used to verify the efficacy and specificity of the β2.1 morpholino. Embryos injected with morpholino showed a reduction in β2.1 mRNA stability (Figure 2.1B). In addition, the morpholino was specific to β2.1 and did not detectably reduce the size or stability of mRNA for the closely related β2.2 subunit (Figure 2.1B). Injection of 750uM of β2.1 (32ng total) morpholino resulted in cardiac defects in 70% of injected

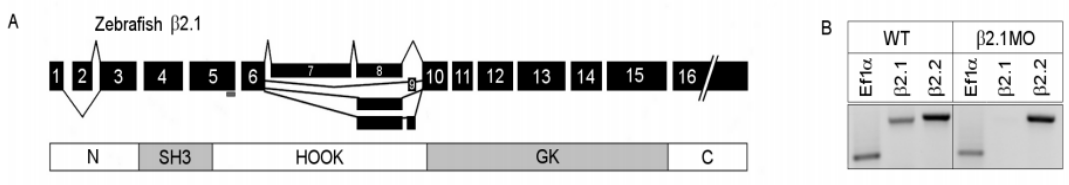

Figure 2.1: Validation of the β2.1 morpholino.

(A) Exon map of zebrafish β2.1 protein showing sites of alternative splicing and MAGUK domains (Ebert et al., 2008c). The SH3 and GK domains (gray) are conserved in all MAGUK proteins. Morpholino binding site is located on the 3' end of exon 5. B) RT-PCR to assess the efficacy of the β2.1 morpholino in reducing full-length *β2.1* mRNA. In RNA from a wildtype embryo (left), *β2.1* and *β2.2* transcripts (each detected with gene-specific primers) are present at 48hpf. In RNA extracted from 48hpf embryos injected with 750uM β2.1 morpholino (right), *β2.1* transcripts were not detectable, whereas expression of *β2.2* transcript levels were indistinguishable from wildtype. The resulting amplicons are 200bp (*Ef1a*), 457bp (*β2.1)* and 472bp (*β2.2*).

embryos. With this dose, off-target effects such as cell death throughout the CNS or altered body axes were observed in fewer than 5% of the embryos.

We also designed a splice blocking morpholino to target β2.2 in order to determine if these two closely related genes had any shared functions. However, we never saw a reduction in mRNA or any evidence of mis-splicing for β2.2 as assayed by RT-PCR, therefore those experiments were discontinued. Please see Appendix 1 for detailed accounts of experiments and methods carried out with β2.2.

Although β2 expression exists at low levels in the brain, it is the predominant β subunit expressed in the adult heart (Ludwig et al., 1997; Ebert et al., 2008c; Zhou et al., 2008b; Link et al., 2009). Therefore we expected that morpholino induced phenotypes might occur primarily in that area. The first overt morphological and functional defects became apparent at ~48hpf when heart looping is well underway in normal embryos. We quantified cardiac looping by measuring the angle of the AV junction (AVJ) relative to the anterior/posterior (A/P) axis of the embryo. Henceforth this measurement is referred to as the Looping Angle (LA). At the onset of looping (~34hpf), wildtype heart tubes displayed an average looping angle of 47˚. Upon completion of looping (~52hpf), wildtype hearts have decreased the average angle to 13˚, reflecting the substantial re-shaping of the heart tube along the A/P axis (Figure 2.2). At 48hpf, roughly 70% of morphant hearts demonstrated a greater average looping angle (50°) between the AVJ and A/P axis than their wildtype counterparts (20°), indicating they were significantly less looped (p=0.009) (Figure 2.3). This phenotype could be rescued in 72% of the embryos by co-injecting wildtype β2.1 mRNA along with the splice blocking morpholino. Morphants were age matched to their wildtype siblings based on fin and otic

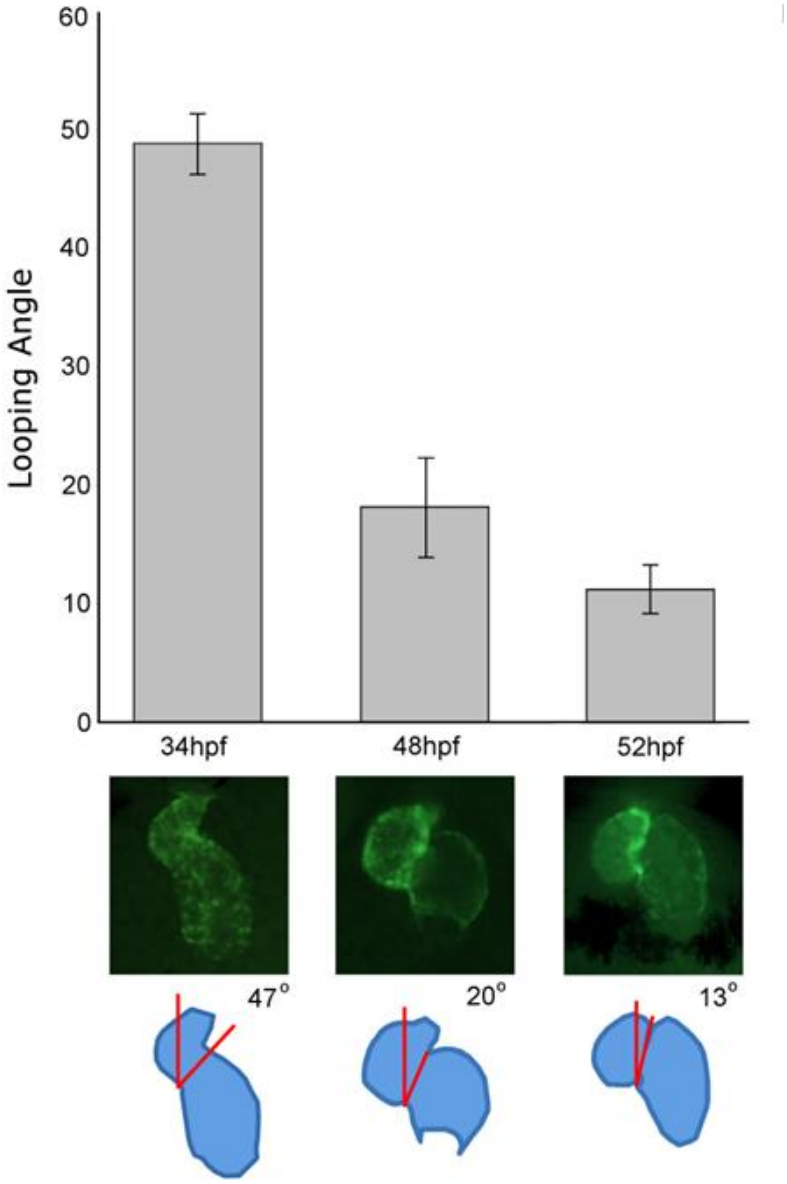

Figure 2.2: Quantification of looping using position of the atrioventricular junction to define the looping angle.

The "looping angle" (LA) is defined as the angle formed between the bisecting plane of the AV junction relative to the anteroposterior axis of the embryo. Tg(myl7:EGFP HsHRAS) hearts were imaged for looping measurements (n=20 embryos/treatment). A schematic layout of each heart with a diagram of the angle formed between the AVJ and the A/P axis is shown below the fluorescent images. In wildtype embryos, the average looping angle decreased significantly as development proceeds.

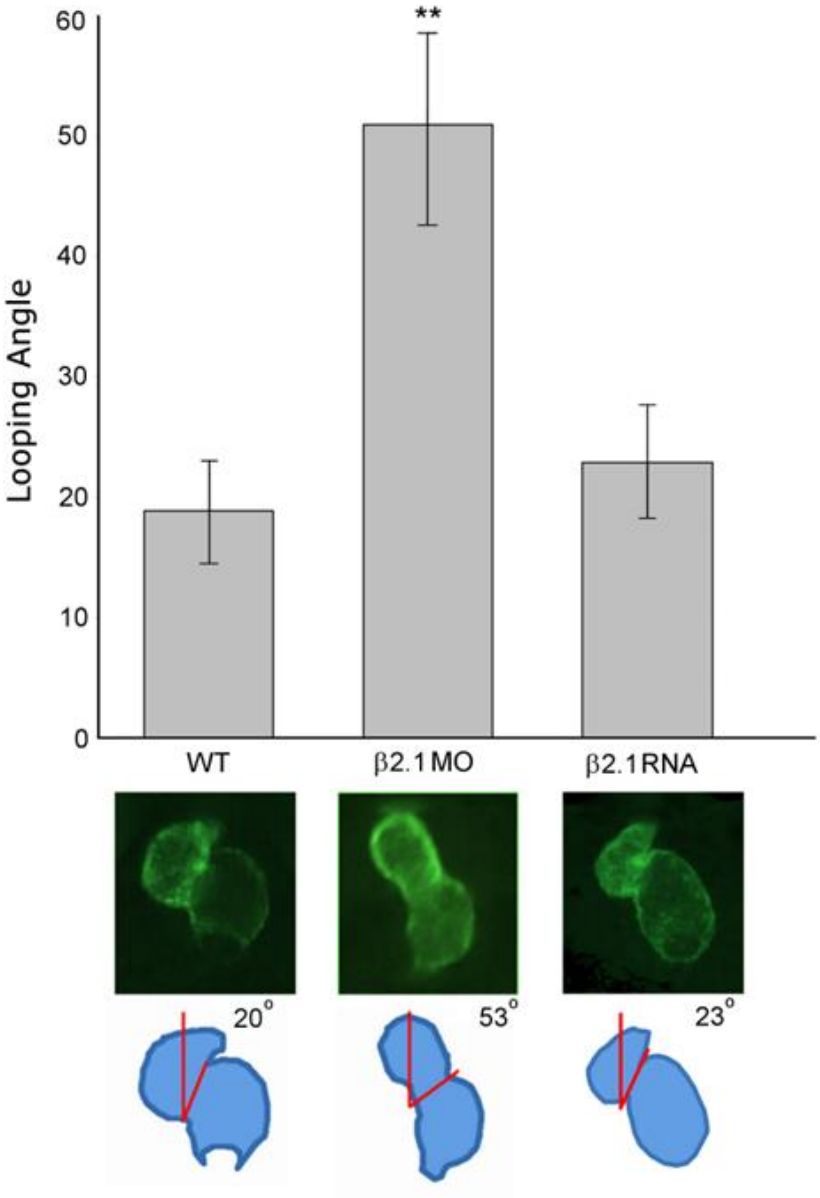

Figure 2.3: LA of wildtype, morphant and β2.1 rescued hearts at 48hpf.

Morphant hearts displayed a significantly greater looping angle (p=0.009; n=20 embryos/treatment). The looping angle was restored to widltype ranges by co-injecting β2.1 RNA along with morpholino.

placode development, indicating that the looping phenotype was not caused by developmental delay. At later stages (72hpf), morphant hearts remained linear and were surrounded by cardiac edema (Figure 2.4).

In zebrafish, cardiac looping occurs concomitantly with chamber ballooning (Auman et al., 2007). During this morphological event the atrial and ventricular chambers acquire their distinct "kidney" shape by differential growth of the cells in the inner (IC) and outer curvature (OC) of each chamber (Auman et al., 2007). OC cardiomyocytes become elongated while IC cardiomyocytes retain a smaller and more rounded shape. Typically, the expression of the curvature marker, *natriuretic protein peptide a* (*nppa*), becomes strongly expressed in the cardiomyocytes of the OC at 48hpf indicating the region of chamber ballooning (Auman et al., 2007). We delineated OC and IC cardiomyocyte regions in morphant hearts according to Auman et. al 2007 (Figure 2.5 II). Individual ventricular myocytes in the OC of β2.1 morphant hearts did not exhibit the usual increase in surface area that accompanies chamber ballooning and looping. Consistent with this observation, morphant OC ventricular myocytes were significantly smaller in size than those of wildtype ($p=0.003$) (Figure 2.5 I, III). Morphologically, the morphant OC ventricular cardiomyocytes resembled the IC cardiomyocytes in their failure to elongate, yet their expression of *nppa* was unchanged (Figure 2.6).

Since looping in β2.1 morphant hearts was severely reduced and cell shape changes that usually accompany ballooning were also decreased, we conclude that chamber morphogenesis does not proceed normally in β2.1 morphants. In addition, contractions of the embryonic heart were weak and blood flow was reduced if not

Figure 2.4: A comparison of wildtype and β2.1 morphant embryos at 72hpf.

Morphant embryos display an overtly wildtype appearance, aside from the large cardiac edema that accompanies their heart defects.

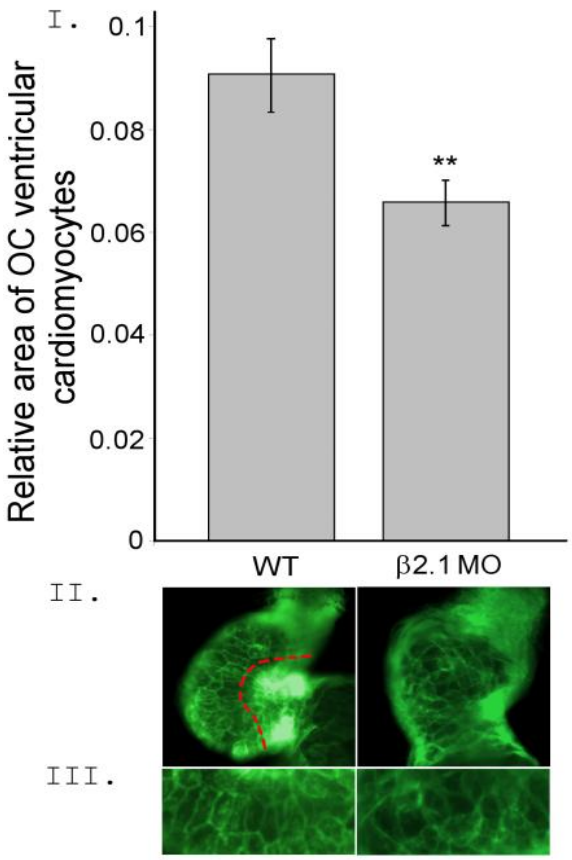

Figure 2.5: Comparison of cell morphology of wildtype and β2.1-depleted cardiomyocytes. (I) Relative area of cells found in the outer curvature (OC) of the wildtype (n=40) and β2.1-depleted hearts (n=36) at 48hpf. Significant differences are notated with asterisks (p=0.003). (II) Tg(myl7:EGFP-HsHRAS) hearts were imaged as the basis for calculating cell area in OC cells. OC cells lie to the left of the red delineation. (III) A magnified view of representative OC cells (from top photos, rotated 90°) showing the elongated shape of wildtype cells compared to the rounder shape of β2.1-depleted cells.

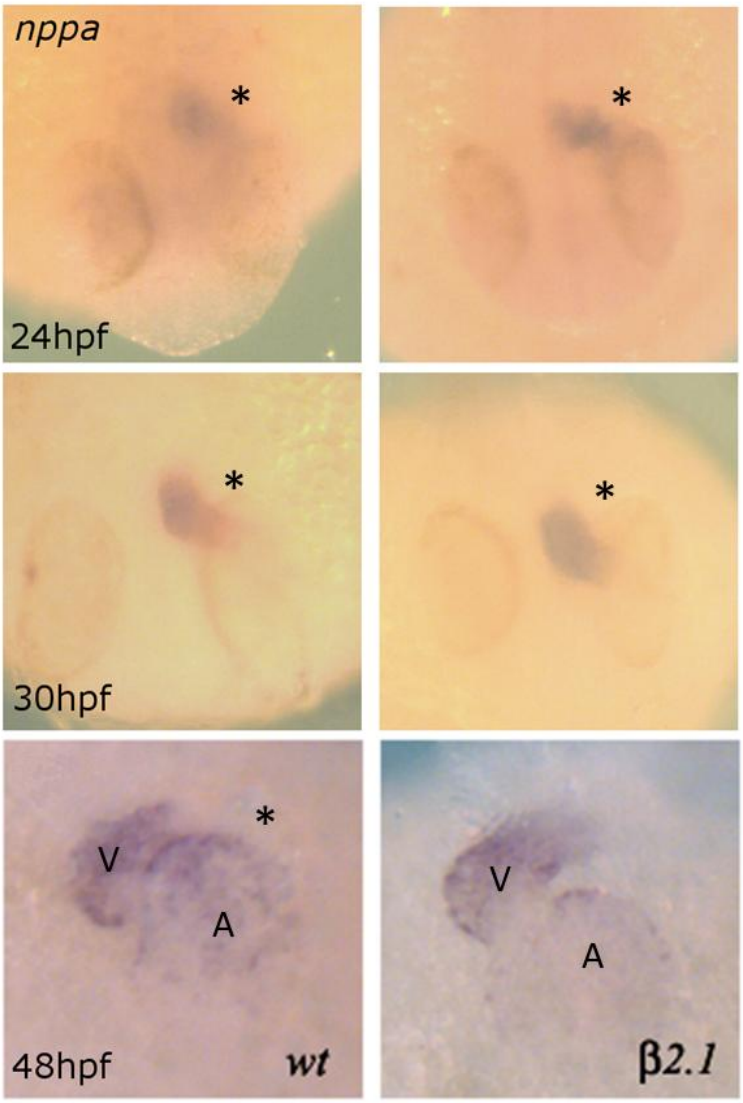

Figure 2.6: Expression of *nppa*, a marker for outer curvature (OC) of cardiomyocytes, in the hearts of wildtype and β2.1 morphant embryos at different stages of cardiac development.

The localization and relative amount of *nppa* in the heart tube remains constant in both wildtype and β2.1-depleted hearts (n=20 for each group and time point). Asterisks denote the left side of the heart. (V) ventricle, (A) atrium.

eliminated altogether. Heart rate was reduced approximately 30% in β2.1 morphant hearts by 48hpf (97 beats per minute [bpm] +/- 3 compared to 120bpm +/- 2 in controls) (Figure 2.7). Thus, cardiac function was compromised in β2.1 morphant hearts. Co-injection of β2.1 mRNA along with the morpholino restored heart rate of morphant embryos to significantly higher than that of wildtype (138bpm +/- 4) (Figure2.7). The offset in heart rates between wildtype and rescued embryos can be attributed to the difficulty of matching protein from injected mRNA to the exact equivalent of endogenously expressed protein.

Although most of the phenotypes observed were in the heart, knockdown of β2.1 affected the development of the central nervous system (CNS) as well. Embryos depleted of β2.1 protein displayed severe hydrocephaly in the mid- and hindbrain regions at 48hpf. This phenotype did not prove to be lethal, since the swelling subsided by 72hpf. Since β2.1 is expressed in the CNS it may play a role in the proper development of the embryonic brain but a detailed study examining that aspect of its function is beyond the scope of this study. Preliminary experiments outlining β2.1 deficiency phenotypes in the CNS are listed in Appendix 2.

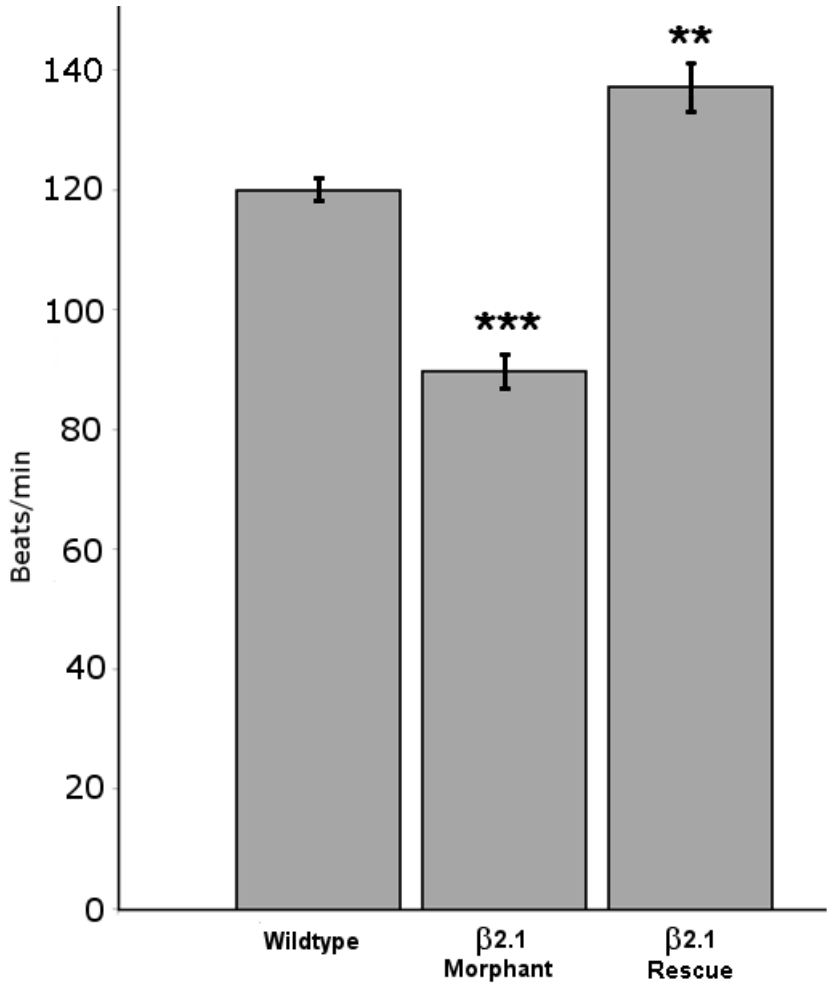

Figure 2.7: Heart rate data in beats per minute (bpm) of wildtype, morphant and β2.1 rescued embryos.

At 48hpf wildtype hearts maintain an average heart beat of 120bpm. The heart rate in β2.1 morphant embryos was significantly decreased to 97bpm on average. Co-injecting β2.1 mRNA along with the morpholino increased the heart rate to 138bpm on average (n=15 for wildtype and rescue, n=20 for morphants) (p=0.002).

Angiogenesis and hematopoeisis are unaffected in β2.1 morphant embryos

A previous report by Weissgerber et al, (2006) described an angiogenesis defect in β2 $^{-/-}$ mice. Although vasculogenesis occurred, the vitelline vessels that should arise by angiogenesis never remodeled into the tree-like network that covers the embryonic yolk sac in wildtype mice by E9.5 – E10.5(Weissgerber et al., 2006). Fewer hemangioblasts were also observed in the β2 $^{-/-}$ embryos. Since the mammalian β2 is not expressed in the yolk sac, the authors hypothesized that the vessel phenotype maybe secondary due to hypoxia. In zebrafish this issue can be circumvented because oxygen needs are met via diffusion. Using a Tg(fli1a:EGFP) line we checked for the presence of angiogenesis. The fli-1 transcription factor is a commonly used marker for endothelial cells that line the lumen of vessels (Brown et al., 2000). In zebrafish, inter-somitic vessels (ISVs) form by angiogenic sprouting in the dorsal trunk of the embryo at two days into development (Isogai et al., 2001). At 48hpf, when cardiac function becomes impaired, the development of ISVs in morphant embryos fully matched that of their wildtype siblings both in size and location (Figure 2.8).

Similarly to what was reported by Weissgerber et al, (2006), we also observed fewer circulating blood cells in β2.1 morphant embryo hearts, which seemed to support the findings that β2$^{-/-}$ mice contain fewer hemangioblasts. To test whether this was due to the development of fewer blood cells or reduction of heart function we washed the embryos in o-dianisidine, a marker for hemoglobin. At 48hpf both wildtype and β2.1 morphant embryos displayed equivalent levels of o-dianisidine staining indicative of blood cells containing hemoglobin (Figure2.9). Although some pooling occurs during the fixation process, the overall amount of blood cells did not appear different between the

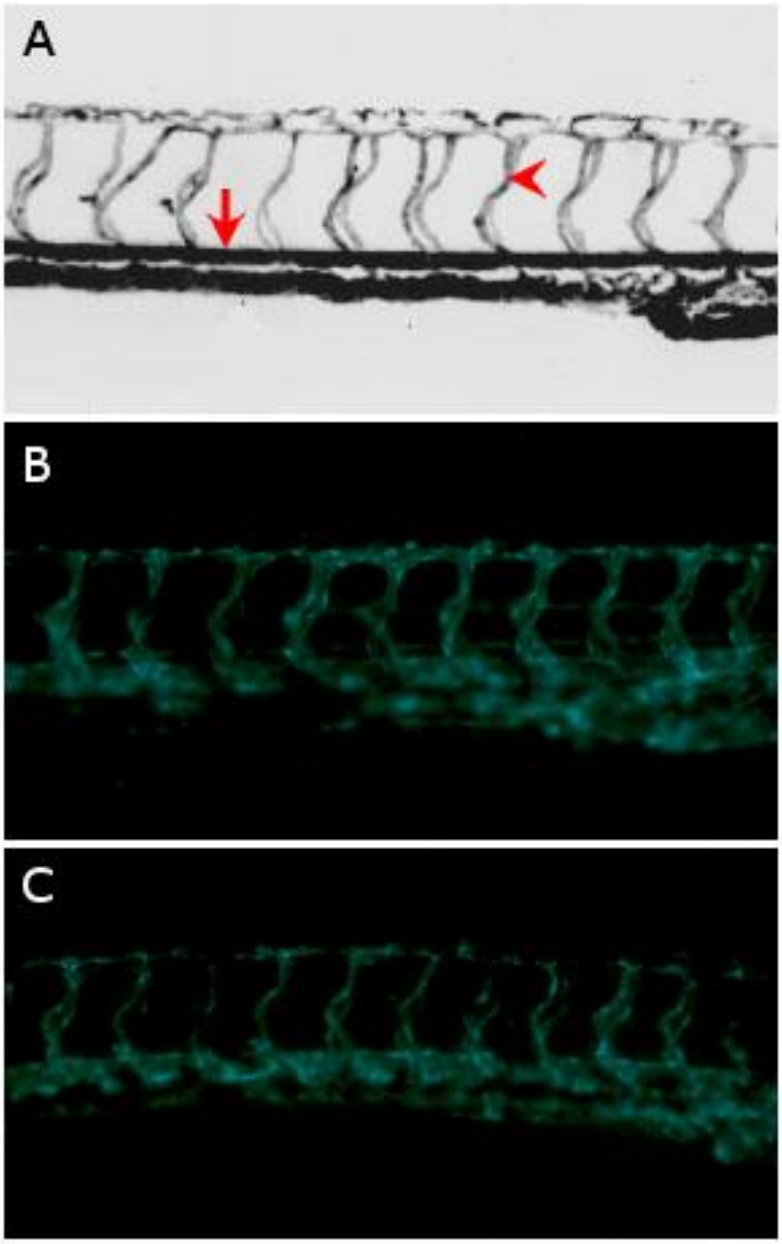

Figure 2.8: Intersegmental vessel (ISV) development in control and β2.1 morhant embryos at 48hpf.

(A) At 48hpf ISVs (arrow head) branch off of the dorsa aorta (arrow) and extend upward between the somites (Isogai et al., 2001) . By 48hpf the ISVs of (B) uninjected and (C) morpholino depleted Tg(fli:eGFP, gata:DsRed) fish invade the inter-somite spaces in a manner similar to wildtype (n=20 for wildtype and morphant).

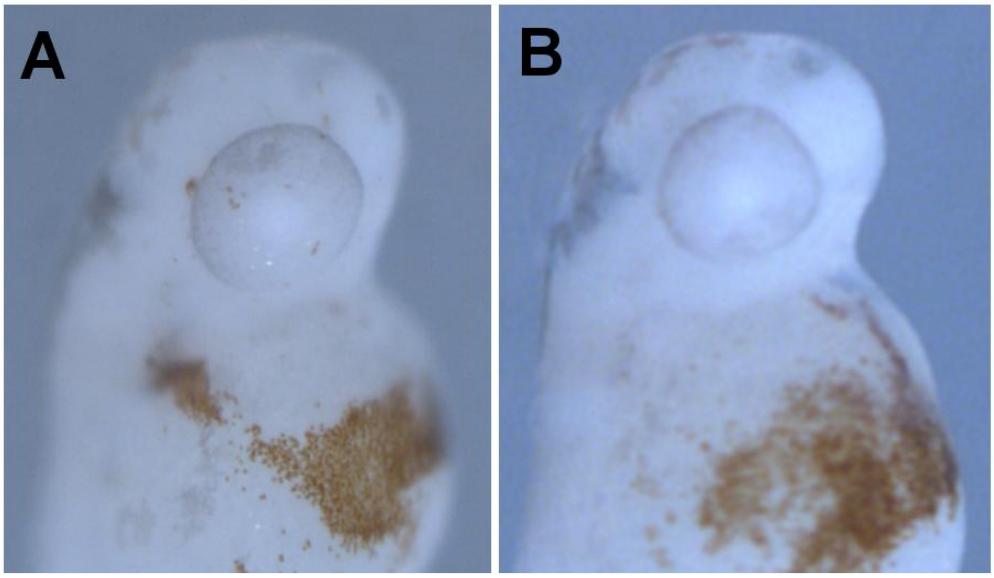

Figure 2.9: O-dianisidine assay to determine the presence of blood in (A) wildtype and (B) β2.1 morphant embryos.

Although not quantitative, the assay shows that blood is present in β2.1 morphants at 48hpf. (n=20 for wildtype and morphant)

two groups. These results confirm that poor vessel development and reduction in hematopoietic precursors observed by Weissgerber et al (2006), was indeed due to impaired cardiac function and oxygen depletion and not a direct role of β2.1 protein in angiogenesis or hematopoeisis.

Cardiac marker expression in the heart field is reduced in β2.1 morphants

In order to investigate the specification and patterning of the cardiomyocyte precursors as a possible basis for the dysmorphic phenotypes observed in β2.1 morphant hearts, we assayed early stage embryos by in situ hybridization using a panel of cardiac-specific markers. *fgf8* is one of the earliest cardiac specification markers expressed and has the ability to induce the expression of other cardiac genes (Alsan and Schultheiss, 2002). The zebrafish *fgf8* mutant *acerebellar* (*ace*) displays a smaller ventricle comparable to that of β2.1 morphant embryos (Davidson et al., 2006; Marques et al., 2008). *fgf8* expression in the wildtype embryo begins prior to gastrulation but becomes localized to the bilateral heart fields during early somitogenesis. We found that at the 4-somite stage, *fgf8* expression in the heart field did not differ between β2.1 morphant and wildtype embryos (Figure 2.10A, B), suggesting that the very earliest stages of cardiac specification occurred normally in β2.1 depleted embryos. Moreover, these data also suggested that the appropriate numbers of cardiac precursors were initially specified in β2.1-depleted hearts.

Next, we examined whether both atrial and ventricular lineages arose appropriately, in slightly older embryos. *cardiac myosin light chain 2* (*myl-7*) is

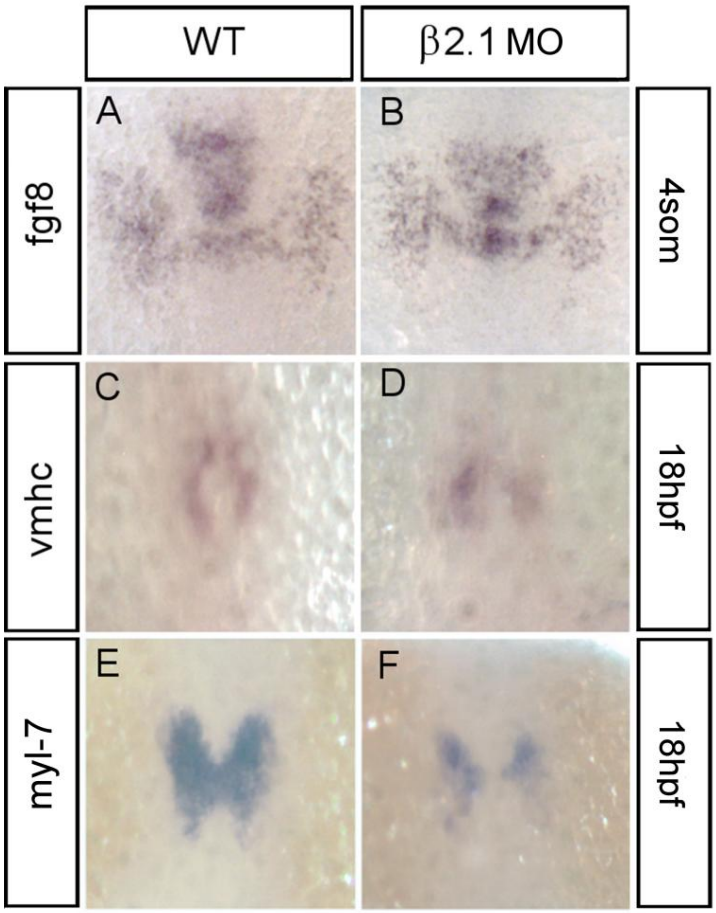

Figure 2.10: Expression of early cardiac markers in the developing heart field. (A,B) Fgf8 expression in the bilateral heart fields at 4som (11.5hpf) is comparable in wild-type and morphant embryos. In contrast, by 18hpf, expression of (C,D) vmhc and (E,F) myl-7 is reduced in the fusing heart fields in β2.1-depleted embryos.

expressed in all cardiac precursors whereas *ventricular myosin heavy chain* (*vmhc*) is expressed only in the ventricular lineages, which are located closest to the midline in the fusing bilateral heart fields (Yelon, 2001). At the 18-somite stage, the region of expression for both of these markers was reduced in β2.1 morphant heart fields (Figure 2.10, C-F).

Since we found no difference in *fgf8* expression between wildtype and morphant hearts but observed a reduction in heart field we next tested the expression of *gata4*. The *gata4* transcription factor regulates multiple genes necessary for cardiomyocyte specification, differentiation and signaling (Durocher et al., 1997; Zhang et al., 2003). *gata4*, along with *nkx2.5*, is an early marker for cardiogenic cells but continues to be expressed throughout the heart tube later in development (Durocher et al., 1997). Depleting embryos of *gata4* can lead to smaller hearts in mild cases and cardia bifida in more severe ones (Zhang et al., 2003). β2.1 morphant embryos did not exhibit a reduction of *gata4* expression in the early heart tube (24hpf) (Figure 2.11).

Thus, even though the heart field was reduced in size cardiomyocytes were able to assemble an overtly normal heart tube. Both the atrial and ventricular lineages appeared to be proportionally represented, suggesting both lineages were specified appropriately. The smaller domains of *myl-7* and *vmhc* expression in morphant versus wildtype hearts suggested that by stages just prior to fusion into the cardiac cone, β2.1 morphant embryos contained fewer cardiomyocyte precursors than wildtype embryos.

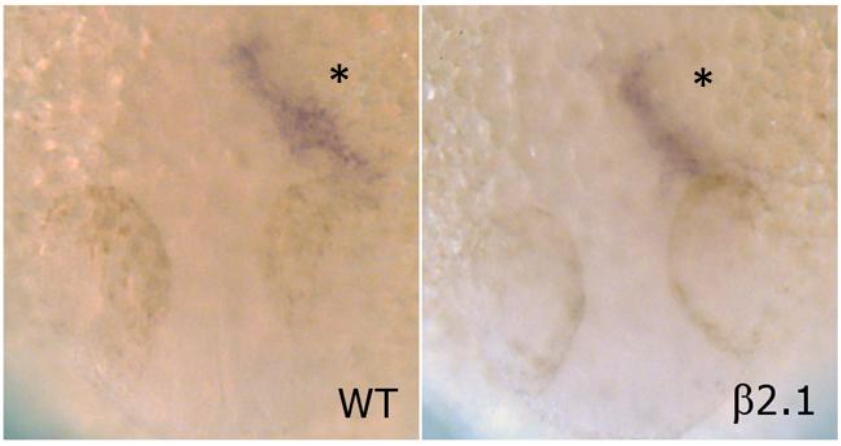

Figure 2.11: GATA-4 expression in wildtype and morphant embryos at 24hpf.

The expression of *gata4* in the embryonic heart tube is similiar in morphant and wildtype embryos (n=20). Asterisks denote left side of heart.

Morphant hearts contain fewer cardiomyocytes due to a reduction in rate of proliferation

Despite an apparently smaller field of cardiac precursor cells, β2.1 depleted embryos did form overtly normal heart tubes at 24hpf. However, hearts of β2.1 morphant embryos subsequently developed morphological aberrancies by 48hpf. Therefore to determine whether reduced cardiac numbers contributed to this phenotype, we quantified the total population of cardiomyocytes in each cardiac chamber at 48hpf. A Tg(myl7:nDsRed2) transgenic line allowed the visualization of cardiac myocytes. At 48hpf, morphant hearts contained 25% fewer cardiomyocytes in the ventricle than wildtype hearts (n=20 per genotype) (Figure 2.12). In contrast, the atria did not exhibit asignificant difference in cardiomyocyte number between wildtype and morphant atria.

We next examined whether the decrease in cardiomyocyte numbers in β2.1 morphant hearts was due to apoptosis or to a decrease in rate of cell proliferation. Using TUNEL assays, we detected very few apoptotic cells in either wildtype or morphant hearts (Figure 2.13). As a positive control, a heart treated with DNAse to induce DNA fragmentation recognized by the terminal deoxynucleotidyl transferase, resulted in large numbers of apoptotic cells. Therefore, cell death did not appear to account for the reduced cardiomyocyte numbers in morphant hearts

Next we assayed for mitotically active cells by BrdU-uptake within 6 hour windows ranging from 24hpf to 48hpf. β2.1-depleted hearts exhibited significantly fewer proliferative cardiomyocytes during early looping stages (24-36hpf) and during chamber ballooning (42-48hpf) (Figure. 2.14). However, embryos did not exhibit any difference in the rate of mitosis at 36-42hpf. These data together with the cell counts in transgenic lines

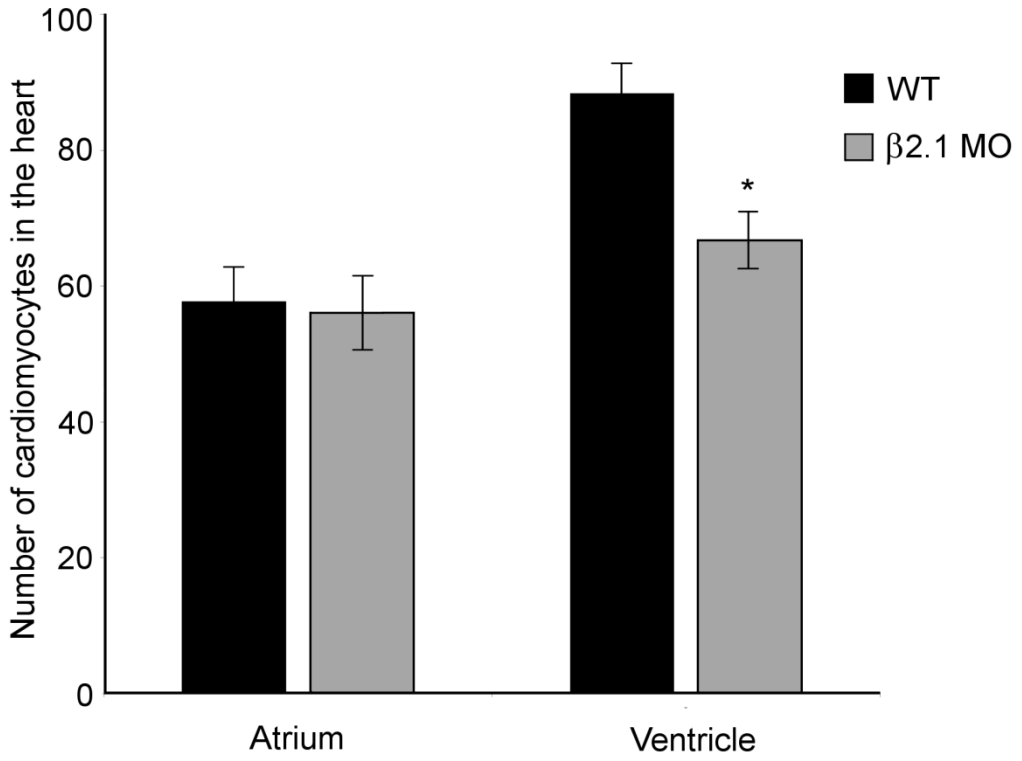

Figure 2.12: Comparison of cardiomyocyte populations in wild-type and β2.1-depleted heart chambers at 48hpf, based on counts of dsRED-labeled nuclei (n=20). Significance is designated by asterisks (p=0.02)

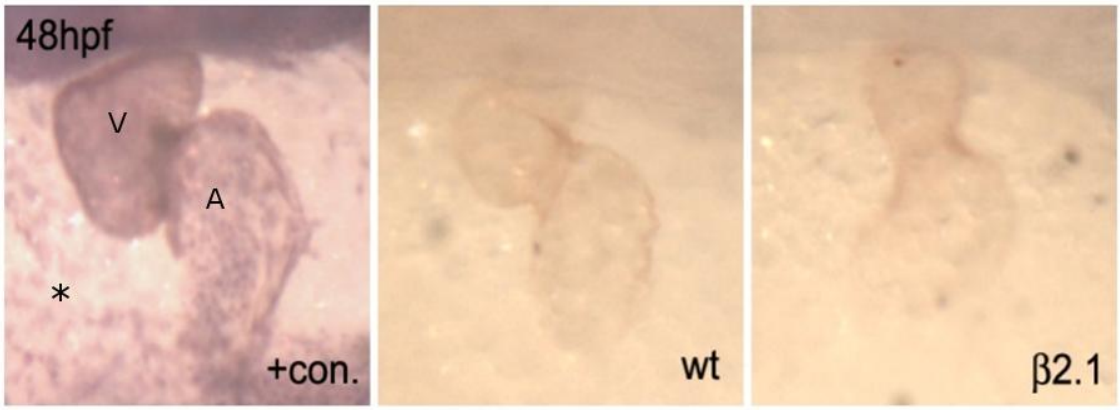

Figure 2.13: A TUNEL assay for apoptosis was carried out on wildtype and β2.1 depleted embryos.

The atrium (A) and ventricle (V) were co-labeled with MF20 antibody for cardiac myosin. A DNAse treated positive control (+con) shows the signal displayed by apoptotic cells.No difference in number of apoptotic cells was observed between wildtype and morphant embryos. (n=20). The yolk is denoted with the asterisk (*).

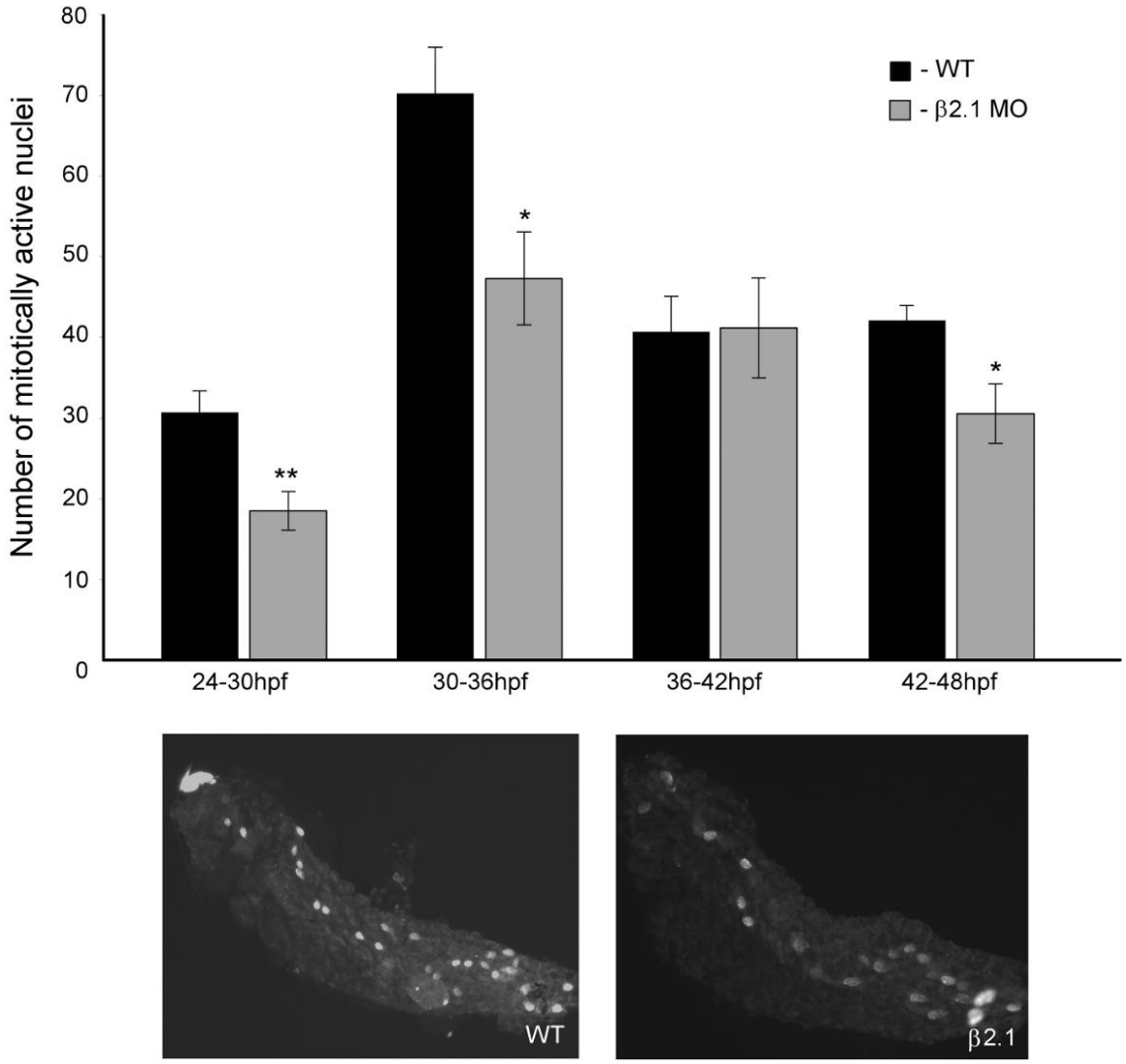

Figure 2.14: BrdU assay of cardiomyocyte mitosis spanning selected periods of cardiac development. β2.1-depleted embryos display a significantly reduced rate of mitosis (asterisks) at 24-30hpf (p=0.002), 30-36hpf (p=0.01) and 42-48hpf (p=0.01). Representative BrdU labeled hearts are shown panels below.

suggest that depletion of β2.1 does lead to a reduction in the number of cardiomyocytes in the ventricle, and that the mechanism of β2.1 action involves regulation of the mitotic cycle at several stages of cardiac development.

In order to determine which signaling mechanism may be responsible for the decreased proliferation of cardiomyocytes in β2.1 morphant hearts we probed for the expression of *bmp4* in the myocardium at 24hpf and 48hpf. *bmp4* regulates the transition between dividing cardiac progenitors and differentiating cardiomyocytes (Wang et al., 2010). More *bmp4* expression would imply that fewer cells are remaining in the cardiogenic progenitor state. At 24hpf wildtype *bmp4* expression was evenly distributed throughout both chambers but increased slightly on the left side of the heart tube (Figure 2.15). This gradient determines the direction of cardiac jogging (Chen et al., 1997; Chocron et al., 2007; Monteiro et al., 2008). The heart tube is predicted to jog towards the side with the greatest *bmp4* expression. By 48hpf *bmp4* becomes restricted to the myocardium that directly overlies the newly forming cardiac cushions. At this time, proliferation at the AVJ is restricted and differentiation is underway as the endocardial cells undergo the endothelial-mesenchymal transition (EMT) that is vital to the formation of properly functioning valves (Figure 2.15) (McCulley et al., 2008; Wang et al., 2010). We found that expression of *bmp4* was up-regulated in the heart tubes in 17 of 20 β2.1-depleted embryos at 24hpf (Figure 2.15). However, the gradient of *bmp4* remains in place consistent with the lack of jogging phenotypes observed in morphants. By 48hpf, when chamber expression of *bmp4* should be down regulated, morphants continue to express it in the ventricle (Figure 2.15). The relative amount of *bmp4* expression at the AVJ does not differ in the morphant hearts compared to wildtype. In support of this observation we

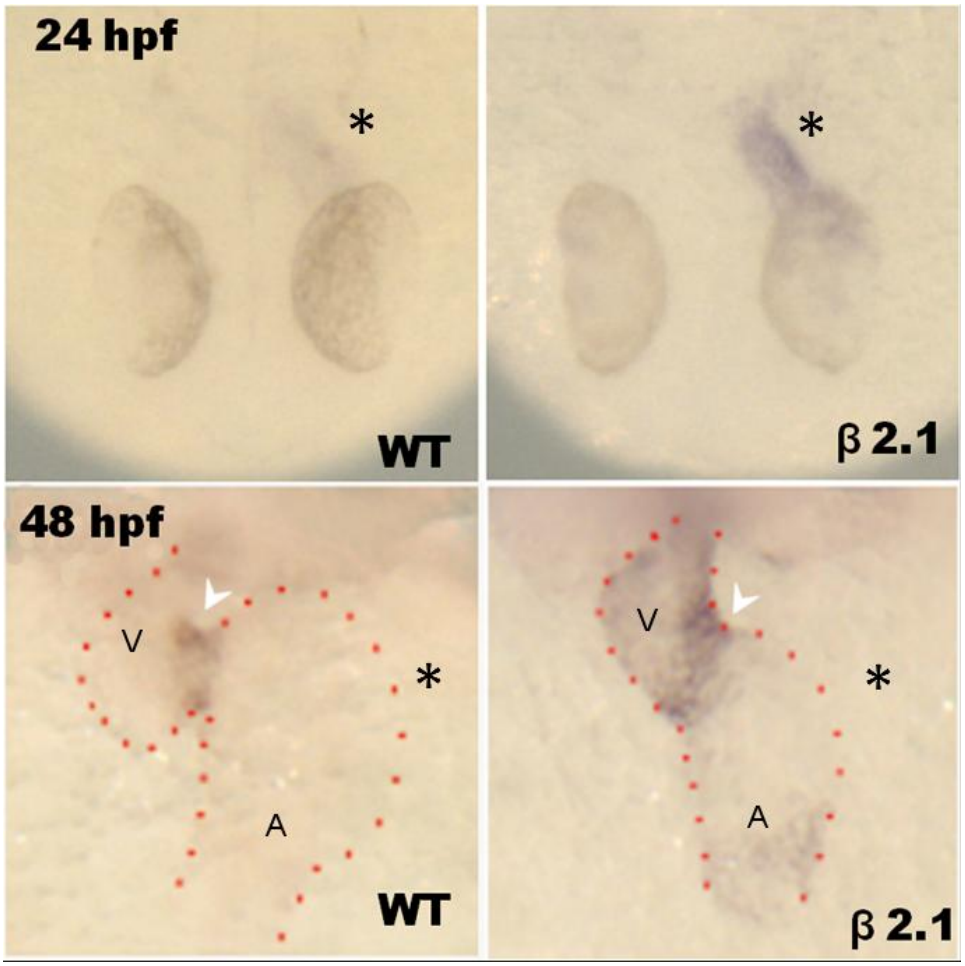

Figure 2.15: Expression of *bmp4* in wildtype and β2.1 morphant hearts at 24hpf and 48hpf.

bmp4 expression is increased in the heart tube of β2.1-depleted embryos at 24hpf compared to the wildtype. At 48hpf when wildtype expression become restricted to the AVJ (white arrow head) morphant hearts show exogenous *bmp4* expression in the ventricle. Asterisk denotes left side of the heart; (A) atrium; (V) ventricle.

failed to see any endocardial cushion defects in Tg(*fli*:eGFP, *gata*:DsRed) embryos at 48hpf (not shown). The exogenous expression of *bmp4* in the heart tube and ventricles displayed by morphant embryos suggests that β2.1 plays a role in limiting the amount of *bmp4* the cardiomyocytes.

Cell adhesion is compromised in β2.1 morphant hearts

Following heart tube formation at 26 hpf, contractions in the embryonic heart increase in force and frequency over the next few days of embryonic development (Stainier et al., 1993; Baker et al., 1997). The continued development of cardiac morphology and cardiac function requires that cardiomyocytes form and maintain complex internal cytoskeletal structures as well as appropriate cell-to-cell connections (Luo and Radice, 2003; Bagatto et al., 2006). To test the structural integrity of heart tubes, we observed their ability to withstand force applied by overlaying a glass coverslip *in situ*. Under these conditions, wildtype heart tubes flattened but remained intact. In contrast, the slight mechanical pressure of the coverslip caused morphant heart tubes to fragment and the cells to disperse and lyse in 30% of the embryos (Figure 2.16). These data suggest that morphant hearts are comprised of cells with an inherently weaker cellular ultra-structure or less robust intercellular attachments.

To further investigate the integrity of morphant cardiomyocytes, we examined the structure of the actin cytoskeleton. In differentiating cardiomyocytes, cellular actin is

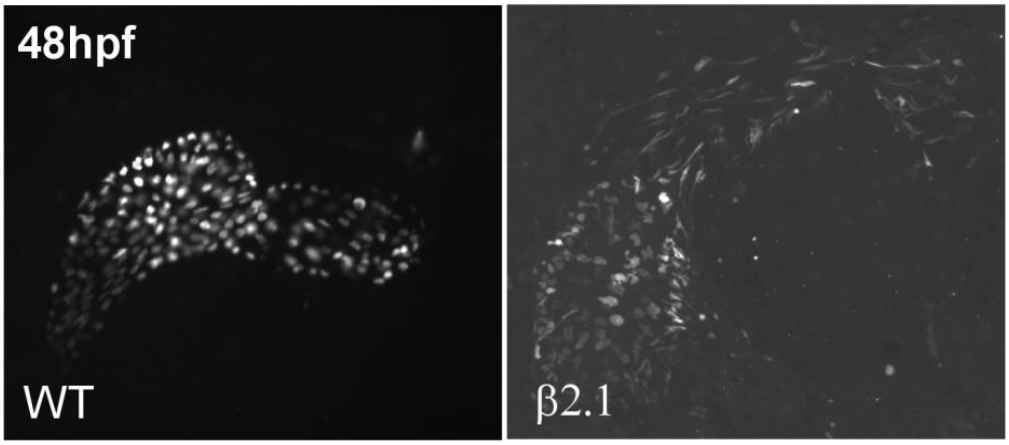

Figure 2.16: Heart presses illustrating heart tube integrity in wild-type versus β2.1-depleted heart tubes at 48hpf. Tg(myl7:nDsRed2) embryos were subjected to slight pressure from a coverslip to determine how well the heart was able to withstand stress. β2.1 hearts dissociated much more easily than wild-type heart subjected to the same pressure (n=20).

assembled into stress fibers that spread beneath the sarcolemma, lending structural rigidity and shape to cells (Pollard and Cooper, 2009). Actin is also a necessary component of the sarcomere without which muscular contraction would be impossible. We examined cytoplasmic actin in cardiomyocytes using rhodamine-labeled phalloidin in order to determine if stress fibers were reduced or misaligned in morphant hearts. Wildtype embryos at 48hpf of cardiac development contained stress fibers that ran the length of the cell below the plasma membrane (Figure 2.17A). The Tg(myl7:EGFP-HsHRAS) transgenic line, which exhibits GFP localized at the plasma membrane, provided a visualization of the cell periphery (Figure 2.17A'). Additionally, actin was clearly present in regular condensed units characteristic of sarcomeres (Figure 2.17A arrow) (Huang et al., 2009). Sarcomeric staining localized around the periphery of the cells in the characteristic pattern of myofibril development (Huang et al., 2009). In β2.1-depleted hearts, stress fiber localization and organization at the cell periphery was indistinguishable from that of wildtype hearts; however, β2.1 morphant hearts failed to display the characteristic banded pattern of sarcomeric actin, suggesting that sarcomeres in morphant cardiomyocytes were fewer or less organized (Figure 2.17B, B').

Within the embryonic heart, adherens junctions are considered to be the most "load bearing" of cellular attachments between neighboring cells (Noorman et al., 2009). N-cadherin (*cdh2*) is the major classical cadherin that has been identified in adherens junctions of cardiac cells (Noorman et al., 2009). N-cadherin mediates calcium-dependent homophilic cell adhesion. The cytoplasmic domain of the cadherin protein is directly linked to the actin cytoskeleton via scaffolding proteins, including MAGUK family proteins (Lilien et al., 2002; Nechiporuk et al., 2007). Some evidence suggests that

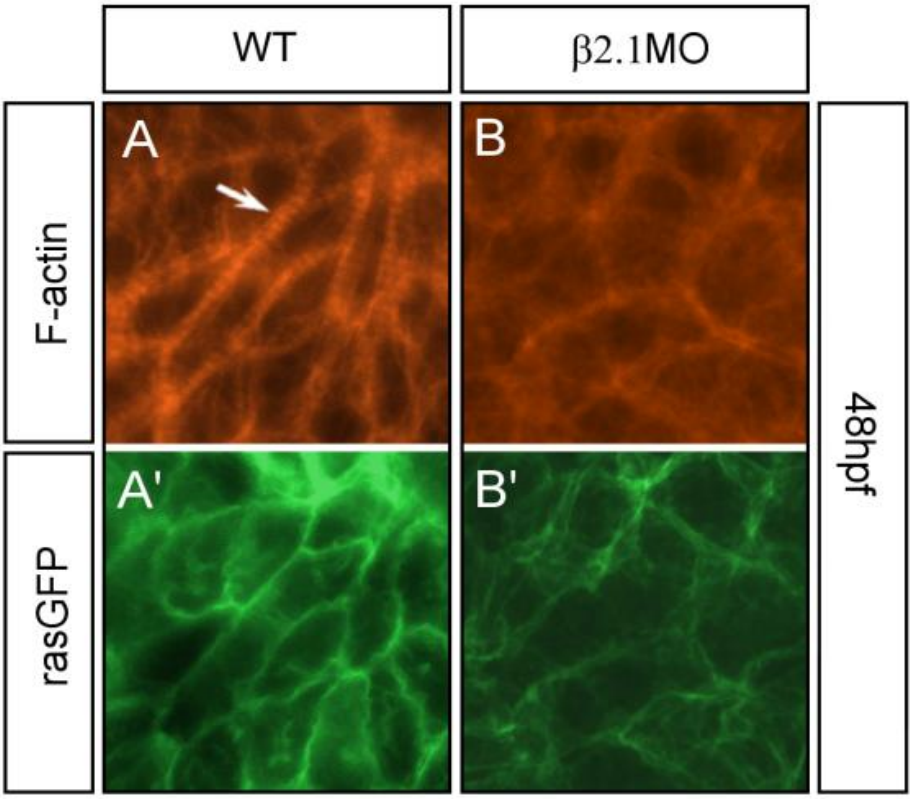

Figure 2.17: Organization and presence of cytoskeletal structures in wild-type and β2.1-depleted ventricular cardiomyocytes.

(A, A') Wild-type cardiomyocytes contain cytoskeletal actin that localizes near the cell membrane at 48hpf. Some of the actin present is condensed and arranged within the sarcomeres (arrow). (B, B') While morphant cardiomyocytes still show the presence of cytoskeletal actin at the membrane the presence of sarcomeric actin is not as prevalent.

myofibrillogenesis in embryonic cardiomyocytes depends on N-cadherin. Specifically, the disruption of N-cadherin function can inhibit the expression of sarcomeric proteins such as α-actinin and myosin (MacGrogan et al., 2011).

We assayed the distribution of N-cadherin protein at cell membranes of wildtype and β2.1-depleted hearts using an anti pan-cadherin antibody in immunohistochemistry experiments. N-cadherin protein localized uniformly near the cell periphery of the cardiomyocytes in wildtype hearts, similar to the expression of membrane associated GFP in the Tg(myl7:EGFP HsHRAS) line (Figure 2.18A, A'). In contrast, β2.1-depleted hearts displayed substantially less N-cadherin localized to the cell periphery (Figure 2.18B, B'). Overall, 66% of β2.1-depleted hearts showed a strong reduction in N-cadherin signal localized at the cell periphery (n=18). Although we observed a reduction of N-cadherin protein, the amount of N-cadherin mRNA did not change between the wildtype and β2.1-depleted hearts (Figure 2.18). Taken together, our data suggests that the presence of β2.1 in the cell impacts the amount of N-cadherin protein localized to the cell periphery. We propose that this in turn adversely affects the maintenance of adherens junctions and integrity of the heart tube.

β2.1 subcellular localization

The preceding work demonstrates that β2.1 is vital for proper differentiation and adhesion of ventricular cardiomyocytes. Yet it was still unclear whether this function was due to a direct interaction of the β subunit with novel binding partners or if β2.1 was indirectly influencing development via its regulation of L-type calcium channels. Since

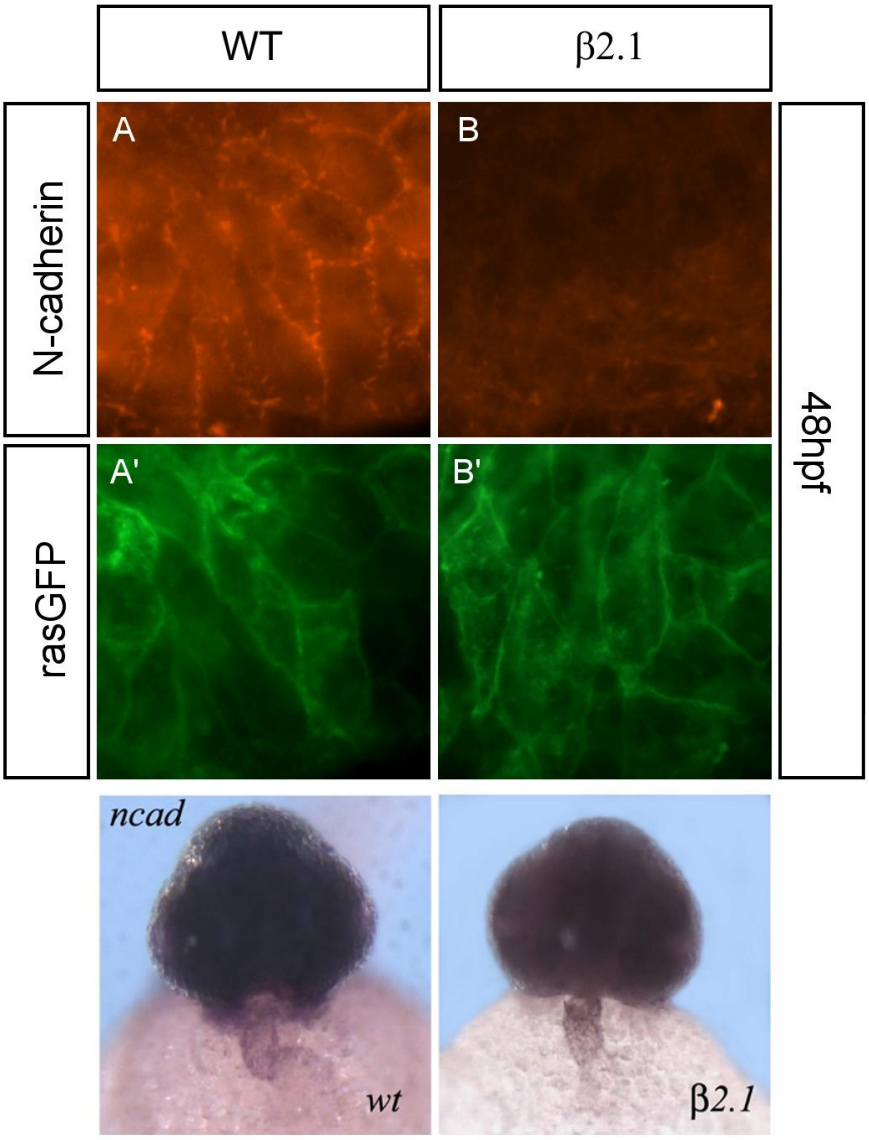

Figure 2.18: Organization and presence of adherens junctions in wild-type and β2.1-depleted ventricular cardiomyocytes.

(A,A') Wildtype cells maintain intra-cellular cohesion by expressing N-cadherin at the membrane (co-labeled with rasGFP), while (B, B') β2.1-depleted cells lack substantial N-cadherin expression at their cell membranes. The depletion in N-cadherin is not due to a decrease in gene expression since both wildtype and morphant embryos still show the presence of N-cadherin mRNA as assayed by ISH.

β2 is necessary for expression of L-type calcium channels at the membrane, some or all of the observed phenotypes could have been the result of a calcium signaling deficiency. In order to determine if this was the case we proceeded to mutate those residues of β2.1 alpha binding pocket that are necessary for α1 subunit interaction. The following three mutations were introduced and verified by sequencing; M213A, L217A, L359A. Transcript variant 6 (tv_6: EU301440) was used for wildtype and mutant β2.1 RNA and cDNA. Henceforth, the mutant β2.1 is designated as mβ2.1. In order to observe β2.1 localization in the cell we fused it to GFP at its C-terminus.

Initially, experiments with the β2.1GFP or mβ2.1GFP fusion proteins were carried out by injecting RNA for each construct into zebrafish embryos and observing fluorescence. Although we successfully observed fluorescence, the dosage of mRNA required to do so (1ug/ul) caused severe morphological defects in developing embryos. Therefore, we chose to make two conditionally expressed constructs for mβ2.1GFP and the control β2.1GFP and inject them as linearized DNA. Both fusion proteins were placed under the control of a heat shock promoter and fused in frame to GFP at the C-terminus (Figure 2.19).

Following heat shock, fluorescence could be observed within 2hrs. All embryos that displayed fluorescence expressed it in a mosaic manner. We observed β2.1:GFP fusion protein predominantly in skeletal myocytes but its expression extended to a variety of cell types (Figure 2.19) (Appendix 3). Being able to visualize β2.1:GFP fusion protein provided a means to compare the sub-cellular localization of wildtype and mβ2.1 protein. β2.1:GFP appeared as a striated expression pattern in most skeletal myocytes at 48hpf (Figure 2.19). This pattern of expression was similar to the localization of the zebrafish

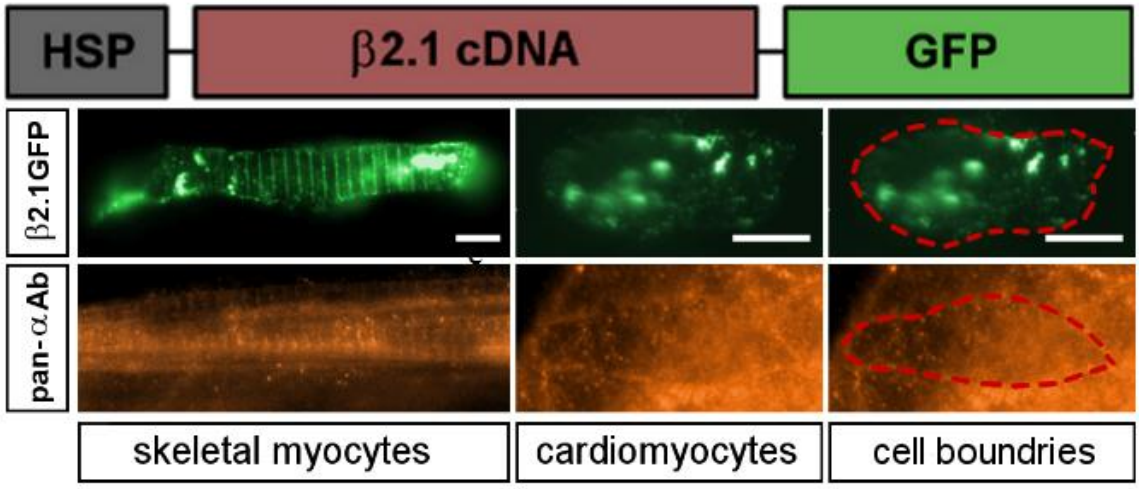

Figure 2.19: Diagram of β2.1:GFP fusion construct and its expression in skeletal or cardiac myocytes at 48hpf.

β2.1 cDNA is under the control of the heat shock promoter (HSP) and fused to a GFP at its C-terminus. When expressed in a mosaic manner, β2.1:GFP localizes to the t-tubules in skeletal myocytes while retaining a punctate expression in cardiac myocytes. Both patterns of expression for the fusion protein coincide with the localization of the α1 subunit of the L-type calcium channel. Scale bars are 1uM.

α1 subunit of the LTCC, as depicted in immunohistochemistry experiments using an anti-Ca_v pan α1antibody. In murine skeletal muscle, LTCC channels were previously shown to cluster at the t-tubules (Jeftinija et al., 2007; Couchoux et al., 2011). The striated t-tubule localization confirmed that the β2.1GFP fusion protein was localizing as expected in skeletal myocytes (Berrow et al., 1995). However, other types of β2.1:GFP distributions were also observed. At 24hpf, we noted that some skeletal myocytes that expressed β2.1:GFP in a dispersed punctate fashion and others that had a diffuse cytoplasmic pattern, without any particular subcellular localization (Appendix 3 and Figure A3.1). At 48hpf the diffuse and punctate localization of β2.1:GFP was still present, albeit at a much lower frequency. In zebrafish, initial membrane invagination, indicative of t-tubule formation, begins at 24hpf, and continues through 96hpf (Zhang et al., 2009). We hypothesize that the transition from punctate to striated β2.1:GFP expression is developmentally regulated, coincident with t-tubule formation.

The mosaic fluorescence of cardiomyocytes was much weaker than that of the skeletal myocytes and occurred less frequently. Unlike mammalian hearts, zebrafish cardiomyocytes lack a network of t-tubules (Shiels and White, 2005; Zhang et al., 2011). Mammalian expression of β2 in cardiomyocytes has been observed at the membrane, but localization to the t-tubules has also been recorded in chicken (Berrow et al., 1995; Colecraft et al., 2002). β2.1:GFP localization in 48hpf hearts (n=7) was always punctate, regardless of developmental timing. The pattern of expression in β2.1:GFP hearts coincided with the punctate expression of the L-type calcium channels (Figure 2.19). Expressing the α subunit of the LTCC in cultured cells lacking t-tubules shows a similar, punctate pattern (Hong et al., 2010). Since the α's localization at the membrane depends

on β subunits trafficking, we presume that the comparable punctate pattern observed for the β2.1:GFP fusion protein indicates its presence at the plasma membrane. However in order to confirm that the GFP expression localized to the membrane cardiomyocytes expressing β2.1:GFP would need to be co-labeled with a membrane marker, or co-localized with the α subunit in the same cell.

The expression pattern of mβ2.1:GFP looked similar to the wildtype β2.1 fusion protein. In fact, using stereo and confocal microscopy we could not detect any difference in the area of subcellular localization between the two fusion constructs. mβ2.1:GFP expressing skeletal myocytes expressed fluorescence in the same striated pattern indicative of t-tubules, with some myocytes showing punctate or diffuse localization (Figure 2.20 A,A' and Appendix 3). In cardiomyocytes, similar to wildtype β2.1, the mutant β expression showed punctate localization (Figure 2.20 B,B'). It is important to note that at no point in time for either β2.1:GFP or mβ2.1:GFP was there any nuclear localization as has been documented for some splice variants of other β subunits (Colecraft et al., 2002; Zhang et al., 2010).

Although further testing is needed to conclude whether mβ2.1 localizes to the membrane in a pattern similar to wildtype β2.1, it is possible that it still interacts with the α1 subunit at a region that is outside the alpha-binding pocket. Conversely, if this interaction is not mediated directly by the α subunit it implies that β2.1 has additional binding partners at the membrane in close proximity to the localization of the L-type calcium channel. In mice the $\beta 2_g$ subunit is palmitoylated and able to localize to the membrane in the absence of any α subunits (Chien et al., 1996). However, those residues required for palmitoylation of mouse β2 are absent from the zebrafish β2.1 ruling out this

post-translational modification as a mode for membrane targeting. Further experimentation is needed to identify β2.1's binding partners and mode of membrane localization.

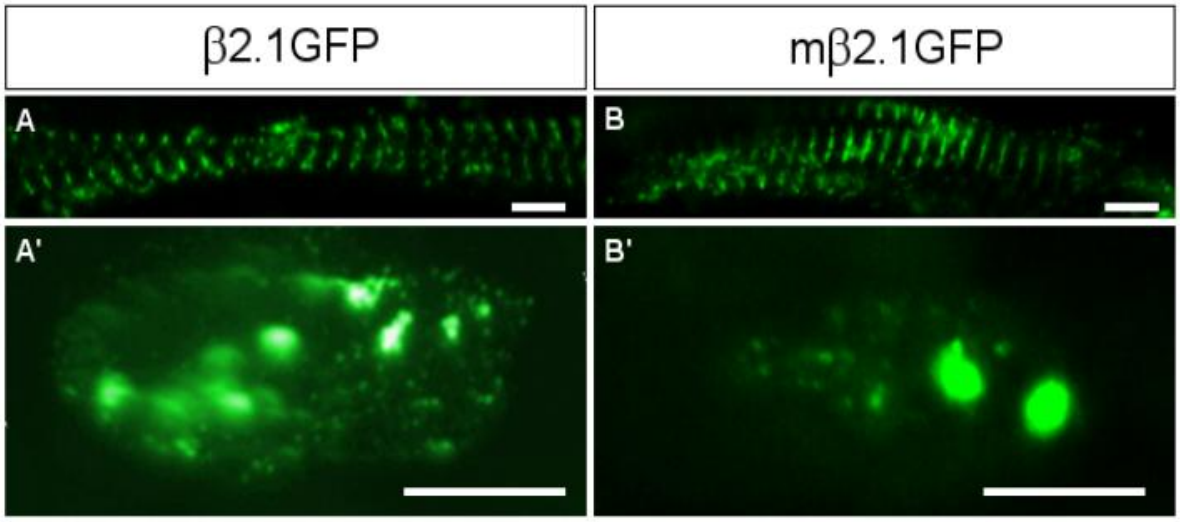

Figure 2.20: General localization of β2.1:GFP and mβ2.1:GFP in skeletal and cardiac myocytes at 48hpf.

(A,A') Wildtype β2.1:GFP shows a striated pattern of expression in skeletal muscle and punctate in cardiac muscle. (B,B') When cells express mβ2.1:GFP the localization pattern is indistinguishable from that of β2.1:GFP. Scare bars are 10uM.

CHAPTER 3: MATERIALS AND METHODS

Microinjection:

Morpholino was injected into the yolk of one-celled embryos using a Femtojet micro-injector (Eppendorf). The volume of the injected droplet was estimated to be 1/8 the total size of the yolk (approximately 30pl). Microinjection needles were pulled using 0.75mm glass capillaries (World Precision Instruments) on a Sutter P97 Flaming/Brown Micropipette puller. The puller setting was as follows:

Heat: 715, Pull:60, Vel:80, Time:200

Injection of wildtype β2.1 RNA for rescue experiments was injected at a concentration of 20ng/ul. The solution was injected directly into the cell of one-celled embryos. Typically, rhodamine was added to the RNA mixture at a final concentration of 0.5ug/ul to assist in selecting well injected embryos.

cDNA for β2.1GFP or mβ2.1GFP was injected at a concentration of 15ng/ul according to our protocol for RNA injections. Embryos were reared at 28° until time of heat shock. Embryos were never heat shocked prior or during epiboly as this led to 100% embryo fatality. Embryos were heat shocked at 24hpf for 1.5hrs at 37°. The earliest GFP signal was observed two hours after heat shock.

Morpholino design and validation:

The β2.1 gene includes multiple translational start sites encoded by alternatively spliced exons at the N terminus (Ebert et al., 2008c). To simultaneously knockdown all known splice variants of β2.1, the morpholino (Gene Tools) was designed to target the splice donor site of exon 5 [MO: 5'-CCACCAGTCATTGTTAAACTTCTGT]. Blocking the donor site is predicted to lead to the joining of exon 4 to exon 6, thus introducing a frameshift and a premature stop codon nine residues into exon 6. The truncated β2.1 protein would lack the GK domain, rendering it unable to interact with the α subunit. Analysis of a dilution series indicated that a dose of 750uM produced the greatest proportion of cardiac phenotypes with a minimum of off-target effects.

RNA was extracted from 48-hour wildtype and β2.1-depleted embryos using Trizol (Sigma). An oligo-dT primer (Invitrogen) was used to generate cDNA, which was subsequently amplified by PCR using β2.1 and β2.2 gene specific primers

[# 74 5'-GCGCAACTCGACAAGGCCAAGAGTA,

#140 5'-TCTCGTTGGACCTTCACTCAAGGGC;

#138 5'-GGGGCCCACTGAGGAGAAGGAGAAGA,

#139 5'-TAAGTGAAGGCCCCATGAGCACCAC] and control primers complimentary to EF1a [#47 5'- CGGTGACAACATGCTGGAGG,

#48 5'- ACCAGTCTCCACACGACCCA].

cRNA Synthesis:

Full-length cRNA of zebrafish *β2.1_tv6* (GenBank#: EU301439) obtained using RT-PCR was used for all rescue experiments. RNA was made using the mMessage Machine RNA kit (Ambion) according to manufacturer's instructions.

Fusion protein construction and mutagenesis:

The full-length β2.1_tv6 cDNA was cloned into the PCR-II (Invitrogen) plasmid vector using TA cloning. It was then sequentially cloned into a PCR-II vector containing GFP followed by excision with restriction enzymes and cloning into PCR-II containing a Heat Shock Promoter (HSP). Injections of linearized cDNA were performed as outlined in previous section. The clone for the wildtype β2.1GFP fusion protein was used as template for the site-directed mutagenesis to eliminate the function of the alpha binding pocket (ABD) on the β2.1 protein. The following phosphorylated primers (IDT) were used in the mutagenesis reaction:

5'GAGGTAACAGATATGGCGCAGAAAGCGGCGTTTGACTTTCTGAAG

5'CTAGATGAGAATCAGGCGGAAGACGCCTGTGAG

Change-IT Multiple Mutation Site Directed Mutagenesis Kit (Affymetrix) was used according to manufacturer's instructions to introduce three mutations as are described in (Ebert et al., 2008a). The mutated residues were as follows: M213A, L217A, L359A. All clones were sequenced to verify the success of the site-directed mutagenesis.

Morphological analyses:

To quantify the reduction in cardiac looping of β2.1 morphants, we compared morpholino-injected and buffer-injected embryos using the Tg(myl7:EGFP) line. At 48hpf embryos were fixed in 4% PFA and mounted in 1% agarose with the heart optimally positioned for imaging. We quantified looping by measuring the angle formed between the anterior/posterior axis of the embryo and the cross-sectional plane of the AV junction. To assess cellular morphology, morphant and wildtype hearts were imaged at 48hpf in the Tg(myl7:EGFP-HsHRAS) line. Cardiomyocytes in the outer curvature of the ventricle were designated according to specifications set by Aumen et. al, 2007. The area of individual cardiomyoctes was measured using the ImageJ software.

Heart presses:

We assessed the integrity of the heart tube in control and β2.1-depleted embryos of the Tg(myl7:nDsRed2) transgenic line. A 48hpf embryo was placed on a slide in a approximately 50ul of E3 media (ZFIN) and a cover slip was gently lowered on top of it to slightly flatten the heart tube. Freshly prepared samples were imaged immediately using a Leica 5500 microscope.

In situ hybridization and immunohistochemistry:

The following digoxigenin (Roche) labeled probes were used for in situ hybridization: *fgf8*, *vmhc*, and *myl7*. Embryos were fixed in 4%PFA for 2hrs and

subjected to permeablization and probe hybridization as previously described (Thisse and Thisse, 2008). Probe signal was detected using the NBT/BCIP chromogenic reagents (Roche).

TUNEL assay was performed according to manufacturer's protocols as per the ApopTagPlus Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Cat# S7101). Embryos were then processed for immunohistochemistry using MF20 antibody to label cardiac specific myosin. BrdU analysis was used to quantify mitotically active cardiomyocytes at different times in development in conjunction with MF20 immunohistochemistry. The embryos were washed in 10mM BrdU reagent (Roche) and then developed for 6hrs. Afterwards they were fixed in DENTS fixative overnight and then rehydrated to PBT. Embryos were treated with Proteinase K to permeabilize tissues, followed by a 10min fixation in PFA and 1hr incubation in 2N HCl. After blocking in BSA and sheep serum embryos were incubated in MF20 (1:50) overnight, followed by overnight incubation in secondary alkaline-phosphatase conjugated anti-mouse (1:3000)(Sigma) and an Alexa488 conjugated anti-BrdU (1:100)(Caltag Labs). Detection of MF20 was achieved with the NBT/BCIP reagent. Hearts were dissected using an Olympus dissecting microscope and imaged for Alexa488 with the Leica 5500 stereo microscope. Images of the flattened hearts were used for counting BrdU positive nuclei. Embryos older than 30hpf were drained of blood prior to fixation to exclude erythrocytes that might have incorporated BrdU.

N-cadherin, the only classical cadherin known to be expressed in the heart, was detected using a pan-cdh Ab (Sigma #C3678) (Noorman et al., 2009). Tg(myl7:EGFP-HsHRAS) control and morpholino injected hearts were dissected after overnight fixation

in 1% formaldehyde. Hearts were subjected to MeOH and PBS-TX washes followed by blocking in PBS-TX containing 1% (w/v) BSA. Samples were incubated in primary anti pan-cdh (1:300) overnight followed by secondary goat anti-rabbit Alexa546 (1:200)(Invitrogen). All fluorescence labeling was imaged using the Leica 5500 fluorescent microscope and IPLab imaging software.

We tested for the presence of L-type calcium channels at the cell membrane using a rabbit anti-Ca_v pan α1(AlamoneLabs #ACC-004) antibody that targets the pore forming α1 subunits of all L-type calcium channels. Embryos were fixed for 1hr in methanol at -20° and then slowly rehydrated to PBT. Then embryos were incubated in chilled acetone for 10min at -20°, followed by three washes in PBT. At this time hearts were dissected out of some embryos and transferred to 24-well plates. The remaining steps were carried out similarly on whole embryos or the excised hearts. Reactions were then blocked for 1hr. Following this the embryos were incubated in primary antibody (1:50) for 2hrs at 30°. The primary was removed and the embryos were rinsed in 1X PBT four times. This was followed by another quick, 15 minute, blocking step (10% sheep serum, 1% DMSO, 0.5% Triton in PBS) and then the addition of the secondary Alexa546 (1:200). Reactions were incubated in the secondary antibody overnight and then washed in 1X PBT. Embryos and hearts were wet mounted on slides and imaged using a Leica 5500 stereo microscope.

O-dianisidine staining for hemoglobin

Two day old morphant or wildtype embryos were dechorionated at fixed overnight in 4%PFA. Fixed embryos were then washed three times for five minutes in 1X PBS and incubated in the staining buffer (0.6 mg/mL o-dianisidine, 10 mM sodium acetate (pH 5.2), 0.65% hydrogen peroxide, and 40% ethanol) for 15min in the dark. The stained embryos were then mounted on 1% agarose plates and imaged using an Olympus dissecting microscope.

Phalloidin staining for f-actin:

Wildtype and β2.1-depleted Tg(myl7:EGFP-HsHRAS) embryos were fixed in 4%PFA. Explanted hearts were washed in PBS-TX twice and blocked in PBT with 1% DMSO and 10% BSA (w/v) for 30min. Rhodamine conjugated phalloidin (Invitrogen #R415) was added in a 1:100 concentration for 30min. Hearts were then rinsed in PBT and imaged with a Zeiss LSM 510 confocal microscope.

Statistics

Histograms present the mean value± SEM. Statistical analysis was performed using ANOVA or Student's t-test. One asterisk represents a significant difference ($P<0.05$), whereas two asterisks represents a highly significant difference ($P<0.001$).

CHAPTER 4: DISCUSSION

β2.1 is an auxiliary calcium channel β subunit expressed in zebrafish embryonic heart (Ebert et al., 2008c). In order to determine the role of β2.1 in cardiac development, we depleted β2.1 gene products in the embryo by morpholino knockdown. Since mammalian β2 is a major regulator of the cardiac LTCC, we predicted its knockdown might lead to impaired cardiac function in the zebrafish embryo. A significantly slower heart rate and weak cardiac contractions in β2.1-depleted embryos confirmed this hypothesis. Here we show that β2.1 is essential for growth of the ventricular chamber, progression of heart tube looping and chamber morphology, and for the structural integrity of the heart tube.

The mechanistic basis for the β2.1–depletion phenotypes involves several components. First, both atrial and ventricular cells are normally specified in β2.1–depleted hearts, and cell survival is normal in both chambers. Next, β2.1 is required for appropriate cardiomyocyte proliferation in the ventricle but not the atrium, throughout most of the period of cardiac looping and chamber morphogenesis. Finally, although cardiac cells in β2.1-depleted embryos appear healthy and generate normal actin stress fibers, N-cadherin is severely depleted from the cell periphery in morphants, suggesting

that the attachments of neighboring cardiac cells via adherens junctions are compromised in these embryos.

β2.1 determines the size and morphology of the ventricle

A major finding of our study is that embryos depleted for β2.1 develop a heart field showing reduced expression of cardiac markers, and later form a heart tube comprised of significantly fewer ventricular cardiomyocytes compared to wildtype. In β2.1-depleted hearts, fewer cardiomyocytes enter the mitotic cycle, as assayed by BrdU. To our knowledge, this is the first report to demonstrate an effect of a β subunit on cell proliferation, but additional studies will be needed define whether this effect is direct or indirect.

When we depleted embryos of β2.1 protein the number of ventricular cardiomyocytes dropped while the expression of *bmp4* in that region increased (Figure 2.14 and 2.15), suggesting that β2.1 may restrict *bmp4* activity in the ventricle by limiting the amount of its transcript present. In support of this hypothesis, it is well established that bmp4 regulates cell proliferation in many contexts (Jiao et al., 2003; Sadlon et al., 2004; Lee et al., 2007; McCulley et al., 2008; Wang et al., 2010). Typically, loss of *bmp4* signaling results in defects that affect the AV region of the heart. Disruption of bmp4 leads to alterations in cardiac cell number in both mice and zebrafish (Lee et al., 2007). *bmp4* null mice have fewer cells in the OFT (McCulley et al., 2008). Zebrafish with a dominant loss of function in the bmp receptor alk8, contain roughly 20% fewer cardiomyocytes (McCulley et al., 2008; Marques and Yelon, 2009). However, other

studies suggest that the mechanism used by *bmp4* to negatively affect proliferation is to suppress cardiac progenitor genes and induce differentiation (Wang et al., 2010). To further investigate the hypothesis that *bmp4* up-regulation results in fewer cardiomyocytes it would be valuable to examine expression of *bmp4* regulators.

β2.1 might affect cell proliferation via several possible mechanisms. Since we observed no nuclear localization of β2.1:GFP in the heart, it is unlikely that it has any direct transcription factor functions (Figure 2.19). In contrast, cell culture and tagging experiments, show that β4 and β3 are able to translocate to the nucleus, suggesting a potential role in gene regulation (Colecraft et al., 2002; Hibino et al., 2003; Zhang et al., 2010). It is more likely that the interactions of β2 which affect cardiomyocyte proliferation are limited to the cytosol and to proteins that are able to translocate to the nucleus. β2.1 could interact with upstream regulators of *bmp4* and thus result in abnormal cardiomyocyte proliferation. These regulators could be directly responsible for controlling cardiomyocyte numbers in addition to *bmp4* expression.

One candidate gene that could lead to the observed increase in levels of *bmp4* expression is the *shox2* transcription factor. *shox2* directly binds to the bmp4 promoter and activates its transcription (Puskaric et al., 2010). The area of *shox2* expression in zebrafish cardiomyocytes at 48hpf overlaps with *bmp4* suggesting a functional connection between the two proteins. (Blaschke et al., 2007; Puskaric et al., 2010). In addition, *shox2* -/- mice have dilated atria similar to β2.1 morphants and upregulated expression of *nkx2.5*, an early marker for cardiac specification (Blaschke et al., 2007). A misregulation of *shox2* in morphant cardiomyocytes could implicate it as a target of β2.1.

Evidence suggests that *bmp4* can regulate cardiomyocyte number in the mouse AVJ, where its disruption leads to valve defects (Jiao et al., 2003; McCulley et al., 2008). However, we never observed malformation of the endocardial cushions in β2.1 morphants as viewed in the Tg(*fli:*eGFP, *gata:*DsRed) line. Although, this could potentially point to a new mechanism for *bmp4* signaling that functions mainly in the ventricular myocardium, the more likely scenario is that the cell decrease phenotype observed in morphants may not be directly the result of increased *bmp4* expression. Other upstream factors exist that regulate *bmp4* in addition to the cell cycle. *gsk3b* limits bmp4 expression in the heart (Lee et al., 2007). Zebrafish *gsk3b* morphants have increased *bmp4* expression at the heart cone stage and ectopic *bmp4* expression in the ventricle at 48hpf. *gsk3b* morphants also have cardiac looping defects (Lee et al., 2007). In addition gsk3b is a major player in controlling the cell cycle by phosphorylating cyclinD (Sklepkiewicz et al., 2011).

The developmental functions for β2 proteins should be considered in light of both calcium channel-related and channel-independent activities. β2 subunits might affect cellular processes through protein:protein interactions mediated by their MAGUK domains, especially the SH3 and GK regions. Precedent exists for MAGUK-family proteins in regulation of epithelial cell proliferation and density through the stabilization of cyclins during mitosis and through interaction with transcription factors necessary to carry out cell division (Balda et al., 2003; Capaldo et al., 2011). Alternatively, as auxiliary subunits to cardiac LTCC, the influence of β2 proteins on the expression and gating of LTCC may impact calcium signaling which subsequently could lead to altered cell proliferation and gene transcription. As one example, calcineurin is a protein

phosphatase responsive to changes in intracellular calcium following signal transduction events. Cardiac-specific mutagenesis of calcineurin in mice led to altered expression of calcium-handling genes in the embryonic heart, and was associated with a reduced number of cardiomyocytes (Maillet et al., 2010). In 2008, Meissner and Noack showed that blocking LTCC conductivity with channel antagonists in cultured human epithelial cells resulted in reduced proliferation, whereas treating with channel agonists rescued the cell number (Meissner and Noack, 2008). Likewise, mutations in zebrafish that reduce LTCC function have been associated with fewer cells in the developing heart tube (Rottbauer et al., 2001). The zebrafish *island beat* (*isl*) allele encodes a null mutation in the cardiac alpha subunit of the Cav1.2, CACNA1C (Rottbauer et al., 2001). Homozygous *isl* mutant embryos contained 43% fewer ventricular cardiomyoctes. The atria of *isl* embryos fibrillate while the ventricles fail to contract at all. Although atrial fibrillation was never observed in β2.1-depleted hearts, contractility was weakened, and heart function compromised. Thus, there is good precedent that LTCC-mediated calcium signaling can be a significant regulator of proliferation, leading us to propose that β2.1 may affect cell proliferation in a calcium channel-dependent fashion. Nevertheless, LTCC activity appeared not to be completely eradicated in β2.1-depleted hearts, since cardiomyocytes do still contract. A possible explanation for this observation is that other β genes expressed in the heart, including β4.1, β4.2 or β2.2, partially compensate for loss of β2.1, so that some LTCC activity remains (Ebert et al., 2008a; Ebert et al., 2008c).

Studies in zebrafish have indicated that multiple mechanisms determine heart size. Initially, a restricted population of cells in the lateral plate mesoderm is specified to become cardiomyocytes (Yelon, 2001). The recruitment of new cardiomyocytes and

regulated cell division, combined with localized changes in cellular morphology, result in the growth and shaping of the developing embryonic heart (de Pater et al., 2009). Genetic analysis suggests that calcium signaling, paracrine communication, and transcription factor signaling cascades are major mechanisms that collectively regulate these morphogenetic processes in the forming heart in early development (Morin et al., 2000; Rottbauer et al., 2001; Ebert et al., 2005; Marques et al., 2008; Caprioli et al., 2011; Suzuki, 2011). In zebrafish, the cardiac mitogenic factor *fgf8* is expressed as early as the 3-somite stage in the incipient heart fields in the lateral plate mesoderm (Reifers et al., 2000). Genetic analysis showed *fgf8* is one of the earliest players involved in specification of cardiomyocytes, and that it is required for the induction and patterning of myocardial precursors (Reifers et al., 2000). Embryos homozygous for *fgf8* mutant alleles showed a decrease in expression of chamber specific markers at the 21-somite stage, and later developed a heart with significantly fewer cells at 48hpf (Marques et al., 2008). Thus, the Fgf8 paracrine factor is also an important early regulator of cardiomyocyte proliferation. In β2.1-depleted embryos, we found no difference in *fgf8* expression at the 4-somite stage, suggesting that the changes responsible for the reduction in cardiomyocytes most likely occur after the initial specification of the heart field. Smaller cardiac-expressing regions were plainly evident prior to formation of the cardiac cone (18hpf). As development progressed, the ventricle appeared to be more strongly impacted by β2.1 depletion than the atrium, since a lessened rate of proliferation was observed in the ventricular chamber at several subsequent stages through 48hpf.

β2.1 maintains adherens junctions at the plasma membrane

Cardiomyocytes that are actively contracting must maintain strong attachments to the ECM and to adjacent cardiomyocytes in order to facilitate mechanotransduction (Noorman et al., 2009). Several emerging studies suggest that cell junction integrity among cardiomyocytes plays important roles in regulating myocyte growth and survival, cardiac morphogenesis, and in establishment or maintenance of the normal cardiac contractile rhythm (Grossmann et al., 2004; Samarel, 2005; Bugorsky et al., 2007; Al-Amoudi and Frangakis, 2008). Although cardiomyocytes express several different cellular junctions, adherens junctions are thought to provide the major structural integrity to the chambers, which are subjected to constant contractions (Noorman et al., 2009). A major component of adherens junctions, the transmembrane protein N-cadherin, is connected to the intracellular actin cytoskeleton via catenins (Ebnet, 2008).The disruption of N-cadherin in the zebrafish *glass onion* mutant led to lethal cardiac phenotypes that depressed both cardiac function and morphology, and resulted in rounder, more loosely associated cardiomyocytes within the heart tube (Bagatto et al., 2006). In general, a large degree of actin remodeling accompanies the maturation of adherens junctions as they connect to the cytoskeleton (de Mendoza et al., 2010). In our study, embryonic hearts depleted of β2.1 protein showed a drastic reduction of N-cadherin protein localized to the cell periphery, suggesting that far fewer adherens junctions were present at the interface of adjacent cardiomyocytes. In a functional assay for adhesion, these same hearts demonstrated reduced ability to maintain tissue integrity.

Others have reported that down-regulation of N-cadherin is associated with cell rounding and inability of cells to extend lengthwise (Knollmann and Roden, 2008; Hara

and Saito, 2009). In wildtype hearts, we observed that cells of the ventricular OC transitioned as expected from a rounded to elongated cell shape around 48hpf, as chamber ballooning and cardiac looping proceeded (Auman et al., 2007). However, in β2.1-depleted hearts, the cells of the OC failed to elongate and instead retained their rounded shape, suggesting that the molecules or cytoskeletal organization needed to make this transition were lacking. In contrast, hearts of β2.1-depleted embryos were not substantially different in their levels or global organization of F-actin, supporting the idea that cytoskeletal organization was not globally altered in hearts lacking β2.1.

The conversion of nascent cell-cell junctions at the plasma membrane into mature linkages between cells requires the appropriate assembly of junction proteins by MAGUKS and other scaffolding proteins (Knollmann and Roden, 2008; Marasco et al., 2009). The assembly of cell-cell junctions is dynamic and regulated by multiple protein complexes depending on the state of maturation (Velthuis et al., 2007; Ebnet, 2008). Cytoplasmic scaffolding proteins, such as plakoglobin, are thought to coordinate a mechanical link between the junction and the actin cytoskeleton (Funke et al., 2005). The zebrafish plakoglobin mutant displays phenotypes similar to what we observed in β2.1-depleted hearts. The unusually small heart fails to loop, showing a down-regulation of cardiac specific markers, *myl-7* and *vmhc* at 48hpf (Martin et al., 2009). The mutant phenotype also includes a reduction in adherens junctions in the heart, which manifests in less N-cadherin at the membrane. In addition, mutant hearts fail to restrict the expression of *bmp4* to the AV region of the heart by 48hpf. The similarity of loss-of-function phenotypes between the plakoglobin mutants and β2.1 morphants is consistent with the

hypothesis that β2.1 is performing a scaffolding function that enhances stability of junctions.

In some cases, recruitment of additional proteins into a mature adherens junction at the membrane is dependent on calcium signaling and calcium oscillations at the membrane. For example, treatment of fibroblasts with calcium channel blockers reduced cadherin-mediated adhesion by 60% (Ko et al., 2001). Thus, it is reasonable to propose that β2.1might affect the integrity of adherens junctions indirectly, via modulation of LTCC calcium signaling. Alternatively, β2.1 protein might act via its MAGUK protein:protein interaction domains to recruit or stabilize proteins needed to establish or maintain adherens junctions.

β2.1 localizes to the membrane in the absence of the ABP

Since β2.1 morphants exhibit a depletion of adherens junctions at the plasma membrane there is reason to believe that β2.1 can interact with membrane-associated proteins. MAGUKs interact with other proteins via the SH3 and GK domains, which are conserved in β2.1, therefore the most likely hypothesis is that interaction between β and a membrane-associated protein would occur via one of these domains. If β2.1 localization at the membrane occurs independently of its interaction with the α1 subunit, then it should retain its membrane localization in the absence of the L-type calcium channel.

We hoped to deplete the heart of all L-type calcium channels using a translation blocking morpholino that targets the cardiac α1C (CACNA1C) subunit. A successful

depletion would phenocopy the *isl (α1C)* mutant, leading to silent ventricles and atrial fibrillation (Rottbauer et al., 2001). However, although we observed some heart defects, our morpholino failed to reproduce the *isl* phenotype. The α1C morphant ventricles contracted, albeit slower, and the atria did not exhibit any fibrillation. Therefore we abandoned that technique for depleting the cell of LTCCs.

As an alternative to the α1C morpholino we used site-directed mutagenesis to generate a recombinant mβ2.1:GFP protein that lacked the three residues in the ABP essential for α1C membrane localization and gating (He et al., 2007). mβ2.1 sequences were fused in frame with GFP to encode the mβ2.1:GFP fusion protein. Thus, although endogenous LTCCs were still present, we predict they would not be capable of interacting with mβ2.1:GFP protein. This strategy is designed so that all of the β2.1 MAGUK domains that could have additional membrane associated functions were still present.

Endogenous β subunits show variability in subcellular localization depending on the cell type and β subunit (Berrow et al., 1995; Colecraft et al., 2002). In skeletal muscle we observed the presence of β subunits beneath the membrane of the transverse tubules while cardiomyocyte expression was more punctate and spaced out. Both patterns of distribution coincide with the expression of the L-type calcium channels for those cell types in mammals (Berrow et al., 1995; Hong et al., 2010). The punctate localization of the anti-pan Ca_v antibody observed in zebrafish is similar to the pattern of IHC labeling in mammals probably indicative of several LTCCs clustered together in lipid rafts (Tsujikawa et al., 2008; Hong et al., 2010; Marques-da-Silva et al., 2010). Surprisingly, the expression of mβ2.1:GFP showed little to no variation from that of β2.1:GFP (Figure

2.20). Cardiomyocytes expressing mβ2.1 retained the punctate pattern of membrane localization, and the same characteristic striations were observed in the skeletal muscle.

We explored the possibility that our observed localization of mβ2.1:GFP at the membrane is still calcium channel-dependent. There is documented evidence showing that in mice $\beta 2_a$ localizes to the membrane in the absence of the LTCCs due to a post translational palmitoylation added onto its N-terminus (Chien et al., 1996). However, the zebrafish β2.1 does not contain any palmitoylation signal in its N-terminus which could account for the observed membrane localization. Another study shows that a short splice variant exists in mice, termed $\beta 2_g$, that lacks most of its MAGUK domains, including the alpha binding domain (ABP), yet is still able to accompany the α1 subunit to the membrane and affect its gating properties (Lao et al., 2008). A 41 amino acid region in the C-terminus of $\beta 2_g$, termed CED, can localize the α to the membrane, presumably at the α1's calmodulin-interacting LA/IQ domain (Bähler and Rhoads, 2002; Lao et al., 2008). This CED region is conserved among all mammalian β2 subunits; however, the zebrafish β2.1 shows no homology to the CED region as analyzed by CLUSTALW and BLAST alignments (Lao et al., 2008). We hypothesize that the persistence of membrane localization after the mutation of the ABP suggests an additional MAGUK function for β2.1. However this does not rule out the possibility that the recombinant mβ2.1:GFP could interact with pre-existing membrane associated α1C subunits that were chaperoned to the membrane by other endogenous β's. The accepted stoichiometry of α/β is 1:1, yet voltage gated calcium channels can persist at the membrane after all β subunits have been removed (Zhang et al., 2008; Buraei and Yang, 2010). This implies that the turnover rate of the α and β are not the same. Thus, even though only one β can interact with the LTCC

at one time, several different β's might potentially associate with the channel over its entire life. A pull- down assay could be used to determine if such an association is occurring in the mβ2.1:GFP mosaic cells.

Conclusion

We show here that β2.1 is essential for cardiac development and function. Specifically, β2.1 is critical for regulation of cardiomyocte proliferation and for the integrity of cardiomyocyte adhesions, mostly likely mediated through adherens junctions. Although potentially indirect, both of these functions represent novel roles for a β subunit.

Although there is evidence that β4 subunits can translocate to the nucleus in both mammalian cells and zebrafish embryos, β2.1 tv_6 was not detected in the nuclei of zebrafish cardiomyocytes. However, this does not rule out the possibility that other existing splice variants of β2.1 maybe able to localize to the nucleus (Ebert et al., 2008a). In the case of mammalian β4, the GK domain must be completely absent for nuclear localization to occur (Hibino et al., 2003). Two β2.1 splice variants exist that are truncated prior to the GK domain, similar to the reported mammalian β4 (Ebert et al., 2008c). Further research, focusing on additional β2.1 splice variants lacking the region encoding the GK domain, is needed to decipher if the phenotypes observed in β2.1 morphants are specific to individual β2.1 isoforms and whether nuclear localization and transcriptional regulation do indeed exist for this protein.

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APPENDIX 1

ROLE OF β2.2 IN CARDIAC DEVELOPMENT

Introduction

Many events must occur for the development of a functional embryonic heart. Morphological changes accompany fluctuations in gene expression which bestow upon the heart its characteristic shape and function (see Chapter 1). The research outlined in this thesis demonstrated the importance of the β2.1 subunit of the L-type calcium channel in regulating some of those events; such as cell proliferation and cardiomyocyte adhesion. However, due to a genome wide duplication, zebrafish possess two genes that encode for β2 subunits and both must be considered in light of the newly documented significance of β2.1 (Jaillon et al., 2004; Ebert et al., 2008c).

Similar to the structure of β2.1, β2.2 possess the conserved SH3 and GK MAGUK domains (Ebert et al., 2008c). Only 14 exons long, β2.2 has two possible splice variants, both in the N-terminus, and shares ~62% homology with the human β2 at the amino acid level – relatively low, compared to the 87% of β2.1 (Figure A1.1) (Ebert et al., 2008b). However, unlike β2.1, β2.2 does share the conserved palmitoylation sequence of the N-terminus cysteines that are also present in the mammalian β2a (Chien et al., 1996; Ebert et al., 2008c). Thus it is possible that like β2a, β2.2 can target to the cell membrane in the absence of the LTCC. Many adult zebrafish tissues express β2.2, but the strongest expression is in the: heart, brain, kidneys, swim bladder and reproductive organs (Ebert et al., 2008c). Since we know that β2.1 is a vital player in cardiac development we wanted to determine if the function of β2.2 was relatively as important.

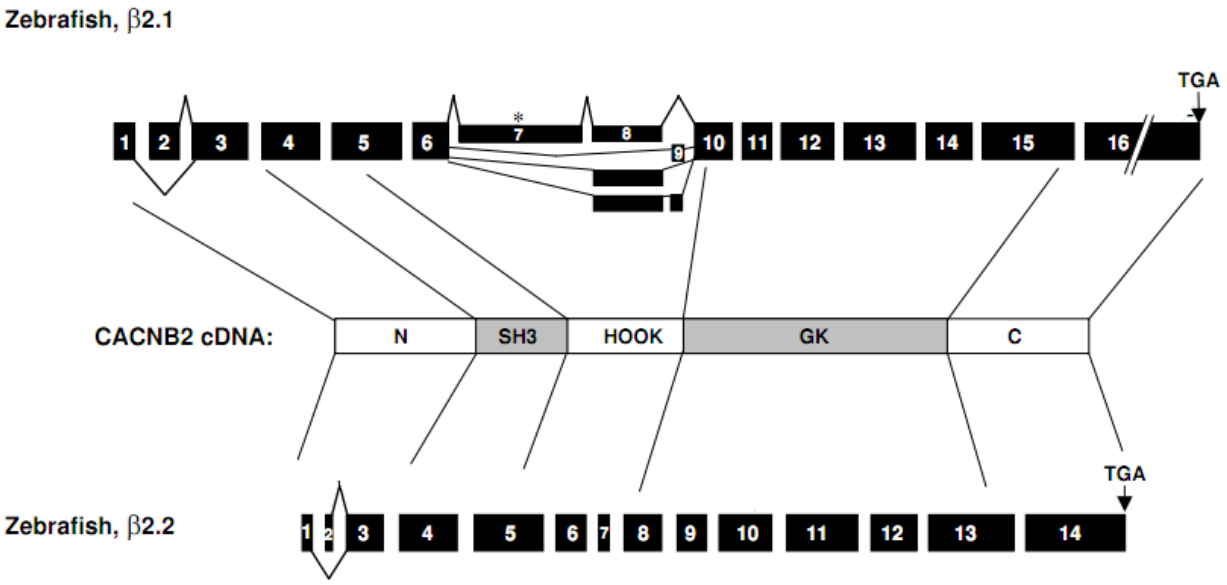

Figure A1.1: Comparison of β2.1 and β2.2 structure and protein domains.

β2.1 retains the higly conserved MAGUK domains (SH3 and GK) but unlike β2.1, has alternative splicing in its HOOK region. Only two possible splice variants are possible from the β2.2 transcript. Adapted from (Ebert et al., 2008c).

β2.2 depletion using anti-sense morpholino

To discover what role β2.2 plays in the development of the vertebrate heart, we depleted its expression using a splice blocking anti-sense morpholino targeted to exon 5. The resulting mis-splicing event would shift the transcript (472bp) out of frame at exon 6 and introduce a premature stop codon three amino acids in. Unfortunately, we were unable to verify the knockdown of β2.2 by RT-PCR, because a shift in transcript size or decrease in its expression was never observed (Figure A1.2). Several other morpholinos were designed in order to accomplish the knock down. Another splice blocking morpholino complimentary to the splice donor site of exon 6 failed to produce any cardiac phenotypes. Two translation blocking morpholinos, co-injected together, also did not produce cardiac phenotypes, although the embryos did exhibit a decrease in hemoglobin as assayed by o-dianisidine. However, after 72hpf hemoglobin staining was ones again observable, leading us to believe that it may have been a non-specific effect of the morpholino injections.

Since zebrafish possess two β2 genes and multiple variations of the β2 subunits it is possible that the phenotype from knockdown of β2.1 is actually more severe than it appears due to compensation from β2.2. Therefore the depletion of β2.2 in an embryo is essential to determining the complete function of β2.1. It is possible that even though no change in transcript is detected in β2.2 morphants less protein is still being made. One possible way to circumvent verification by RT-PCR is to fuse the β2.2 morpholino binding site to GFP and co-inject that with the morpholino. A decrease in fluorescence would indicate that the morpholino is in fact working and is specific to β2.2. Alternatively morpholino injections can be carried out in embryos expressing the whole

β2.2:GFP fusion protein – thus ensuring that interaction of the morpholino with the β2.2:GFP transcript would only happen in vivo and not in the injection solution.

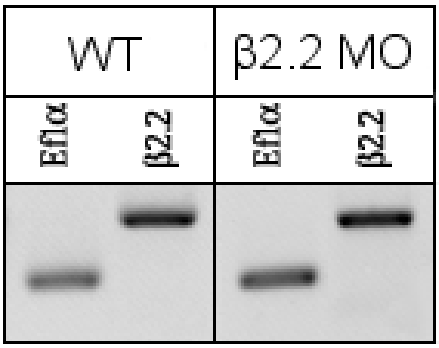

Figure A1.2: Morpholino knockdown verification of β2.2 by RT-PCR.

In wildtype embryos the β2.2 transcript is 472bp long. A splice blocking morpholino at exon 5 should result in the mis-splicing of the β2.2 transcript into a 346bp fragment. In RNA extracted from β2.2 morphants no such fragment was detected and no decrease of the original β2.2 message was observed. Ef1α was used as a control (250bp).

APPENDIX 2

β2 SUBUNIT FUNCTION IN THE DEVELOPMENT OF THE CENTRAL NERVOUS SYSTEM

Introduction

Voltage-gated calcium channels are expressed in all excitable tissues, including the heart and nervous system (Catterall, 2000; Catterall et al., 2007). A variety of L-type calcium channel subtypes occurs in neurons. The different densities and populations of VGCC confer different electrical properties to each neuronal cell type (Forti and Pietrobon, 1993). They are located at the pre- and post synaptic terminal of neuronal cells and serve to regulate multiple functions such as neurotransmitter release, gene expression, synaptic efficacy and activation of other channels (Bading et al., 1993; Lipscombe et al., 2004).

Treating the neuromuscular junction of neonatal rats with calcium channel blockers prevents neurotransmitter release. However, this is dependent on the developmental stage (Urbano et al., 2002). Thus, the roles of VGCC are dynamic in the development and function of synapses of excitable cells. The release of neurotransmitters by VGCC is thought to be mediated by the channels' interaction with various proteins involved in vesicle docking and release, such as synaptotagmin and SNARE complexes (Urbano et al., 2002). This interaction may be as strong as a physical tethering of the neurotransmitter filled vesicle to the site of the VGCC (Figure A2.1) (Rettig et al., 1997; Urbano et al., 2002).

L-type calcium channels (LTCC) in the brain constitute a major source of calcium influx. Calcium entering through the LTCCs, but not other channels, can influence gene expression by initiating signaling cascades that result in transcriptional regulation (Deisseroth et al., 2003). Calcium entering the cell associates with calmodulin which

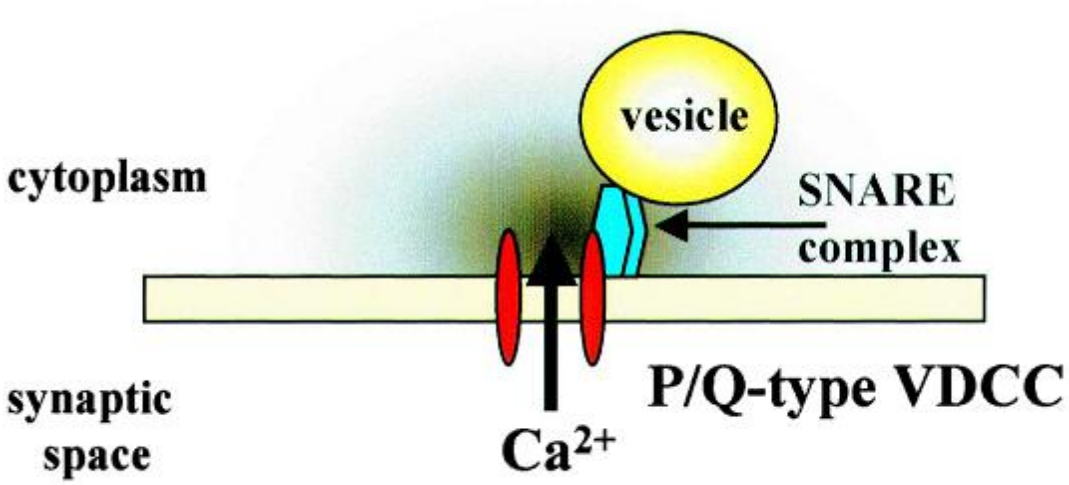

Figure A2.1: Diagrammatic view of LTCC tethering to the neurotransmitter vesicle at the synapse.

The LTCC is directly connected to the neurotransmitter filled vesicle by the SNARE complex. Adapted from (Urbano et al., 2002)

transduces the signal to the nuclear transcription factor NFATc (Dolmetsch et al., 2001; Deisseroth et al., 2003). Additional signaling pathways involving CamKII and MAKP are also possible (Figure A2.2) (Chung and Jan, 2006). The LTCC itself can also serve as a transcriptional regulator since a part of the C-terminus region is able to translocate to the nucleus, in a calcium dependent manner, and interact with RNA polymerase II, potentially regulating its own expression at the membrane (Gomez-Ospina et al., 2006). Since LTCC's show such a diversity in function within the central nervous system (CNS), mechanisms regulating their expression and gating are just as important.

All β subunits are expressed in the CNS to some degree (Buraei and Yang, 2010). Due to the β's promiscuous interaction with multiple VGCC and the fact that numerous alternative splice variants exist for each gene it becomes difficult to gauge which β subunit is the most predominant or the most important for functional neuronal tissue. Never-the-less, using RT-PCR, we have shown that both β2.1 and β2.2 are strongly expressed in the zebrafish embryonic and adult brain, therefore we postulate that the loss of one or both of these proteins may lead to defects in the CNS (Ebert et al., 2008c).

Results

Using antisense morpholino technology we depleted zebrafish embryos of β2.1 protein. Knockdown experiments were performed only with β2.1 due to the difficulty in obtaining a working β2.2 morpholino (See Appendix 1). The CNS of morphant embryos was indistinguishable from wildtype through the first day of development. At 48hpf morphants exhibited a swelling in the regions of the mid and hind brain, particularly in

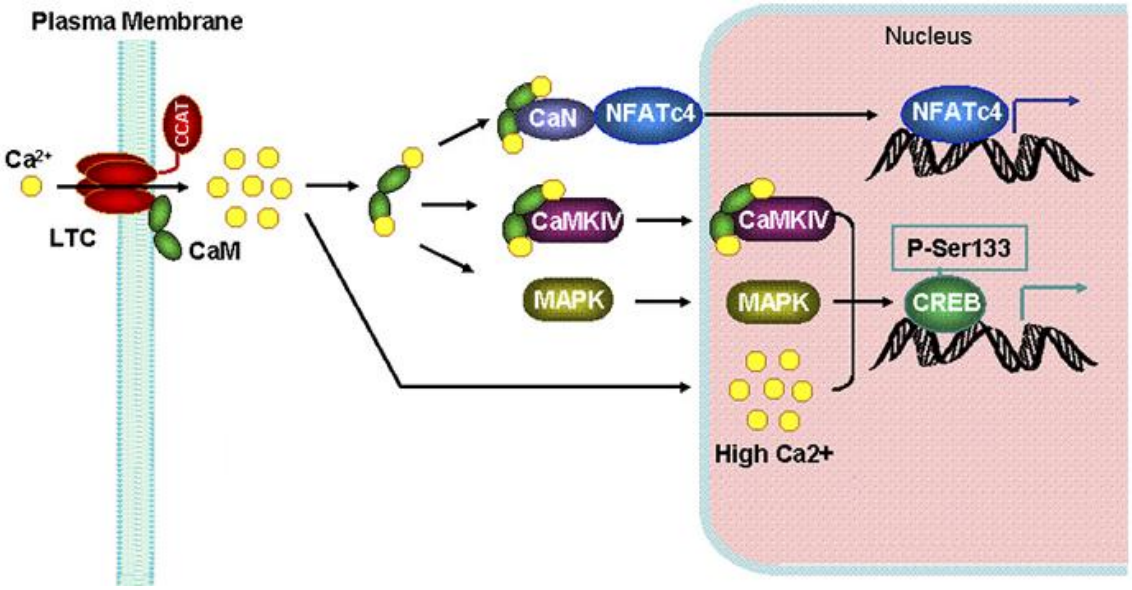

Figure A2.2: Possible signaling cascades that are initiated by Ca^{2+} entry via the LTCC.

Ca2+ entering through the LTCC will associate with calmodulin (green) which will transducer the signal to other transcription factors able to translocate to the nucleus. These in turn will activate the transcription of certain target genes. Adapted from (Chung and Jan, 2006)

the ventricular space (Figure A2.3). As far as we could tell the swelling was the result of an accumulation of fluid in the ventricular space, a condition known as hydrocephaly. The condition did not persist and by 72hpf brain swelling was no longer observed. In situ hybridization using a probe for β2.2 showed that it is localized to the ependymal layer of the mid and hindbrain ventricle (Figure A2.4). The ependyma is responsible for maintaining and producing the cerebra-spinal fluid (CSF) in the ventricles and spine.

To determine what affects, if any, β2.1depletion had on neuronal gene expression and patterning we assayed morphant embryos for the presence of different neuronal markers by *in situ* hybridization and IHC. *pax2.1* is a marker that labels the developing midbrain-hindbrain boundary (MHB) and the neurons of the hindbrain. We detected the presence of *pax2.1* at 48hpf, when hydrocephaly was most prominent. The MHB of morphants showed the same expression of *pax2.1* as wildtype (Figure A2.4). The neurons of the hindbrain also displayed *pax2.1* signal, however, the space in between the two brain hemispheres housing the neurons appeared wider in β2.1 depleted embryos (Figure A2.5), possibly driven apart by the pressure exerted from the hydrocephaly.

Since morphant embryos displayed altered morphology in the region dividing the two hemispheres, we postulated that the defect might extend to the mechanisms responsible for neuronal guidance. Neuronal axons are guided by a combination of long-range diffusible signals and short-range recognition proteins (Diekmann and Stuermer, 2009). Neurolin is a cell surface adhesion molecule essential in the recognition of neurons. Loss of neurolin leads to loss of exonal outgrowths in zebrafish (Diekmann and Stuermer, 2009). At 48hpf wildtype embryos express neurolin in the trigeminal ganglia, pharyngeal pouches and neurons surrounding the otic vesicle (Figure A2.6)

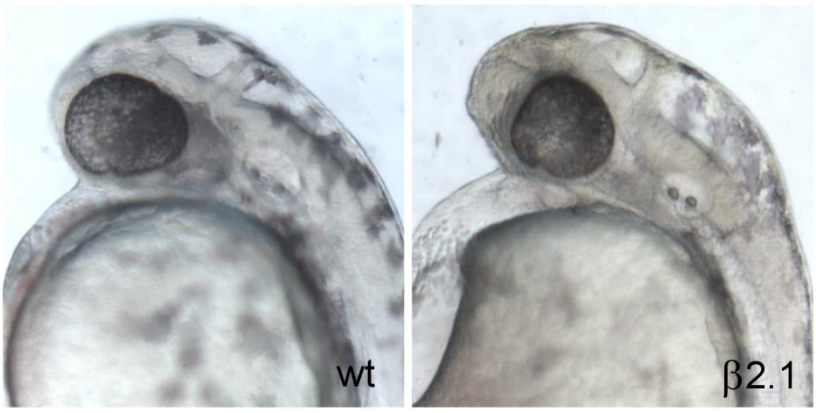

Figure A2.3: Hydrocephalus of the mid and hind brain in embryos depleted of β2.1 protein.

At 40hpf β2.1 morphant embryos developed hydrocephaly in the ventricles of the mid- and hindbrain. The swelling in the hindbrain region was always more prominent.

(Hong et al., 2005). The wildtype axons display neurolin signal in a long continuous, unbroken line. Although neurolin is present, the wildtype pattern of expression is absent in β2.1 morphants. Staining in the endodermal pouches and trigeminal ganglia is completely gone, while the axons above the otic vesicle show a blotchy, disorganized localization (Figure A2.6).

Discussion

It is not surprising that embryos depleted of β2.1 protein displayed hydrocephaly in the mid and hindbrain ventricles at 48hpf since β2 is strongly expressed in the brain (Ebert et al., 2008c; Zhou et al., 2008b). However, all evidence of hydrocephaly was gone by day three. One possible explanation for this is that other β subunits can compensate for the loss of β2.1. The presence of β2.2 in the ependymal layer of the brain implies the presence of L-type calcium channels. Regulation of ionic balance is crucial for osmo-regulation of the CSF. Microvilli found on the ependymal layer are responsible for circulating the CSF. Blocking calcium in that region greatly reduces the beating of the microvilli (Nguyen et al., 2001). L-type calcium channels may be involved in maintaining the calcium concentration across the ependymal membrane, the loss of which causes CSF to build up in the ventricular space.

Morphants also displayed abnormal neuronal guidance, yet the expression of guidance molecules on the surface was still present. It is difficult to draw conclusions about neuronal guidance without further experiments to look at additional cues which mediate axonal growth. Conversely, the disorganized axons of β2.1 brains could be the

result of pressure applied by the swelling ventricles. It would be interesting to see if zebrafish injected into the ventricles with a solution resembling CSF developed the same abnormal neuronal patterning.

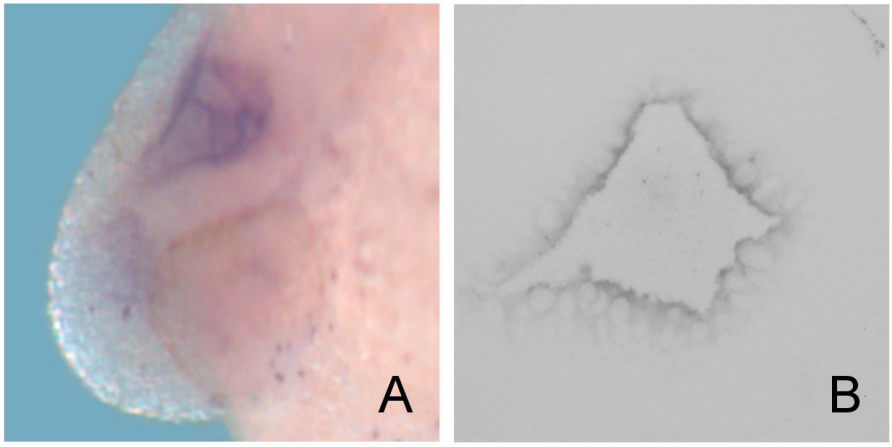

Figure A2.4: In situ hybridization of β2.2 probe at 24hpf in wildtype embryos.

(A) β2.2 mRNA is present in the mid and hindbrain ventricles of the embryonic zebrafish brain. (B) Sectioning through the hindbrain reveals that the signal is specific to the apical surface of the ependymal cell layer.

Figure A2.4: Expression of *pax2.1* in wildtype and β2.1-depleted embryos at 48hpf.

Both wildtype and β2.1 morphant embryos show strong *pax2.1* expression in the midbrain-hindbrain boundary (MHB) and hindbrain neurons. However, morphants display a larger space in between the hemispheres of their brain.

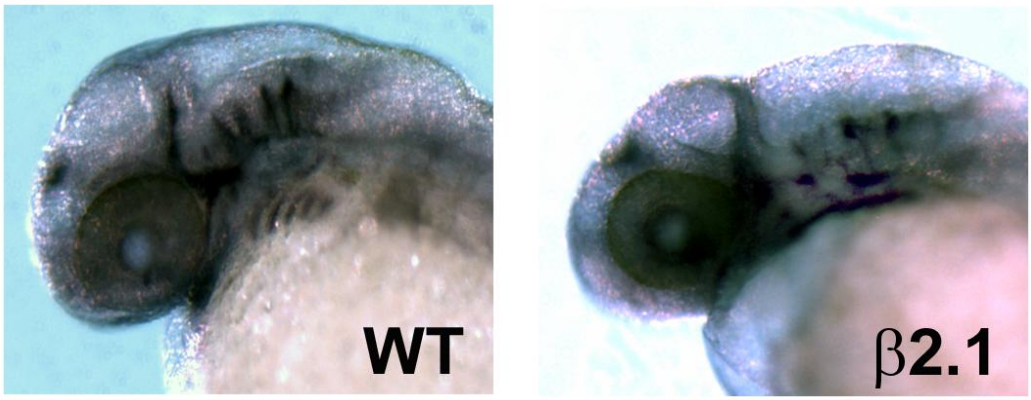

Figure A2.5: Neurolin IHC using the anti ZN-5 Ab in wildtype and β2.1 morphant embryos.

In 48hpf wildtype embryos neurolin is expressed in the developing axons of trigeminal ganglia, endodermal pouches and otic placode neurons. Embryos depleted of β2.1 fail to show neorolin signal on the axonal projections of the trigeminal ganglia and endodermal pouches. Although the neurons dorsal of the otic placode are present their axons display weak neurolin staining and disorganized pattering.

APPENDIX 3

β2.1:GFP EXPRESSION IN DIFFERENT EMBRYONIC TISSUES

Introduction

We created a recombinant β2.1 fused to GFP, under the control of a heat shock promoter, as part of an experiment to determine the localization of native β2.1 in the cell. To our knowledge, this is the first time β2.1:GFP has been expressed in an in vivo system. All previous experiments with β subunits have looked at expression in cultured cells or detected localization using IHC in fixed tissue (Colecraft et al., 2002). Our technique allows us to control the expression of β2.1 and observe its localization in a dynamic cellular environment.

Results

β2.1 expression could be detected about 2 hours after heat shock. Most of the expression was localized to the skeletal myocytes of the trunk. Each myocyte spanned the entire width of the somite. Individual myocytes could be identified without any fluorescence by using the phase contrast setting on the Leica 5500 stereo microscope. In this manner the transverse tubules (t-tubules) of the myocytes could also be observed (not shown). In most skeletal myocytes, β2.1:GFP localized to the t-tubules (Figure A3.1A). This type of localization was also observed for the LTCC (see Chapter 3 Figure 2.19). However, some myocytes displayed diffuse cytoplasmic expression of β2.1 while others showed punctate membrane localization (Figure A3.1B-C).

Occasionally, we were able to detect β2.1:GFP in the cells of the notochord and neurons (Figure A3.2 A-B). The notochord cells form large vacuoles by 48hpf which aide in locomotion and embryo extension by providing the notochord with rigidity (Stemple,

2005; Yamamoto et al., 2010). β2.1 expression at the cell membrane of vacuolated notochord cells was always in a punctate pattern. Strong β2.1:GFP expression in neurons made it difficult to detect any specific subcellular localization. The fluorescence looked diffuse and spread out though the entire cell body and axon.

Some well injected embryos expressed β2.1 in epithelial cells. The epidermis, by far, showed the most varied distribution of β2.1:GFP in the cell. Zebrafish epidermal cells show a textured appearance reminiscent of a fingerprint due to folds in the cell membrane (Horng et al., 2009). These folds were sometimes evident with the expression of β2.1:GFP (Figure A3.1A arrow head). Most epidermal cells expressed β2.1 at the membrane in a punctate fashion (Figure A3.3A). The puncta were small, resembling those observed in cardiac and skeletal myocytes. Occasionally, the puncta would be large, forming flower-like clusters, which sometimes localized around the membranous folds (Figure A3.3B). The degree of β2.1:GFP expression varied considerably from one epidermal cell to another, making it difficult to deduce what the natural pattern of expression would be (Figure A3.3.C).

Discussion

Aside from the notochord, expression of β subunits in muscle, heart, skin and neurons is well documented (Colecraft et al., 2002; Hanson and Smith, 2002; Schjott et al., 2003; Ebert et al., 2008c). Neither β subunits nor L-type calcium channels have ever been shown to exist in the notochord. However, since the notochord cells need to regulate osmotic pressure we can assume that some ion channels exist that carry out this function

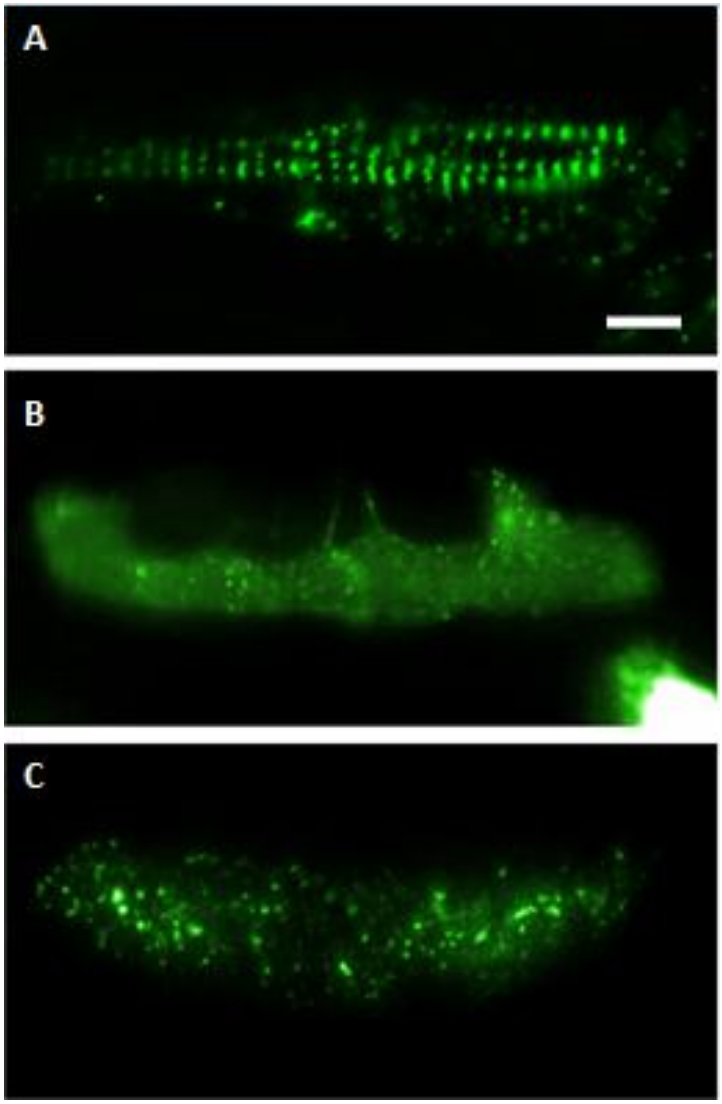

Figure A3.1: Variable localization of β2.1:GFP in skeletal myocytes.

(A) Typical expression of β2.1:GFP in skeletal myocytes is localized to the t-tubules (72hpf). (B) Occasionally, younger embryos, showed a more diffuse β2.1:GFP expression (24hpf), (C) while others looked punctate (48hpf). Scale bar is equivalent to 10uM.

(Yamamoto et al., 2010). Whether β2.1 interacts with these channels and if they are voltage-dependent has yet to be determined. Until endogenous expression of β2.1 in the notochord can be verified it is premature to claim that the β2.1:GFP expression we observed is functional.

Skeletal myocytes and epidermal cells displayed variable subcellular localization of β2.1. It is possible that the changing pattern of β2.1:GFP expression within these cell types is dependent on the developmental stage of that tissue. Skeletal myocytes in zebrafish do not acquire the intricate network of t-tubules till 48hpf, although their formation is evident earlier (Zhang et al., 2009). This could explain why some myocytes failed to localize β2.1 to the t-tubules. Epidermal development is also very dynamic in zebrafish, with rearrangement of cytoskeletal fibers occurring throughout the first 5 days of development (Le Guellec et al., 2004). In addition, its possible that some of the epidermal cells we observed were specialized for other function and therefore displayed different localization of β2.1:GFP (Horng et al., 2009).

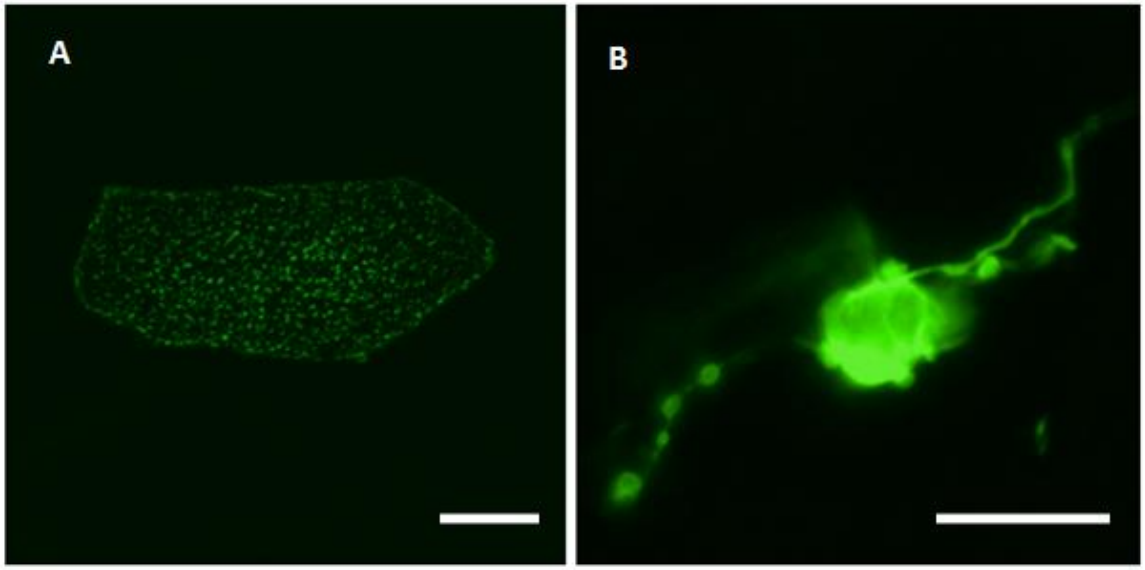

Figure A3.2: Localization of β2.1 in notochord and neuronal tissue at 48hpf.

(A) β2.1:GFP localized to the membrane of vacuolated notochord cells. (B) Expression of β2.1:GFP in neurons was observed in a diffuse pattern throughout the entire cell, including cell body and axon. Scale bar is equivalent to 10uM.

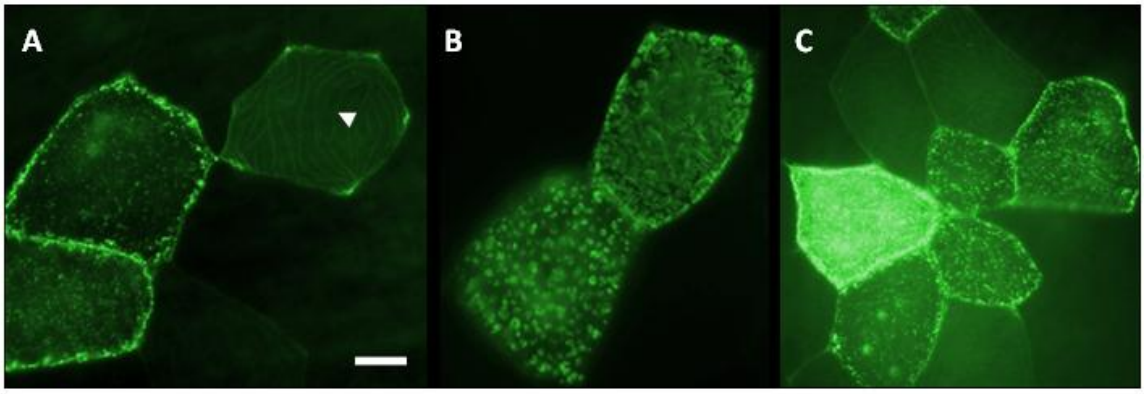

Figure A3.3: Subcellular localization of β2.1:GFP in zebrafish epidermis at 48hpf.

Epidermal expression of β2.1:GFP showed the most diverse subcellular localization. (A) β2.1:GFP was observed in a punctate manner at the membrane, and occasionally around the membranous folds (arrow head). (B) Sometimes the punctate expression was seen as large clusters. (C) The signal intensity with which epidermal cells expressed β2.1:GFP varied from cell to cell. Scale bar is equivalent to 10uM.