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CAMK-II: AN INTEGRAL PROTEIN IN CELL MIGRATION

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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This thesis is dedicated to all my family

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Abbreviations

α	Alpha
ATP	Adenosine Triphosphate
β	Beta
C&E	Convergent & Extension
C-terminus	Carboxy Terminus
Ca^{2+}	Calcium Ion
CaMK-II	Ca^{2+}/CaM Dependent Protein Kinase Type II
CaM	Calmodulin
δ	Delta
DNA	Deoxyribonucleic Acid
Dsh	Dishevelled
FAK	Focal adhesion kinase
FLAG	DYKDDDDK Epitope Tag
Fz	Frizzled
γ	Gamma
g	Gram
GDP	Guanosine diphosphate
GFP	Green Fluorescent Protein
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
Jak	Janus Kinase
JNK	c-Jun N-terminal kinase
MO	Morpholino
n	nano
ncWnt	Non-canonical Wnt
PCP	Planar Cell Polarity
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3-kinase
Rac	Subfamily of Rho GTPases
ROCK	Rho associated Kinase
Ss	Somite stage
μ	Micro

Abstract

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By Jamie J.A. McLeod, Ph.D.

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Coordinated inductive and morphogenetic processes of gastrulation establish the zebrafish body plan. Gastrulation includes massive cell rearrangements to generate the three germ layers and shape the embryonic body. Three modes of cell migration must occur during vertebrate gastrulation and include: epiboly, internalization of the presumptive mesendoderm and convergent extension (C&E). C&E movements narrow the germ layers mediolaterally (convergence) and elongate them anteroposteriorly (extension) to define the embryonic axis. The molecular mechanisms regulating coordinated cell migrations remain poorly understand and studying these has become of great interest to researchers. Understanding cell migration during development is highly relevant to a number of human physiological processes. Abnormal cell migration during early development can lead to congenital defects, with improper cell migration during adult life potentially leading to the invasion and metastasis of cancer. By studying cell migration events, *in vivo*, new insights are to be found to both the function and malfunction of key embryonic and postembryonic migratory events.

The non-canonical Wnt pathway has been identified as an evolutionarily conserved signaling pathway, regulating C&E cell movements during vertebrate gastrulation. With the

absence of the non-canonical Wnts (ncWnts), Wnt5 and Wnt11, during zebrafish development leading to a shorter and broader body axis with defects in elongation during segmentation resulting in undulation of the notochord. While it is clear ncWnts are necessary for C&E, many of the downstream effectors regulating these cell movements have not been defined.

Previous research has shown that activation of ncWnt signaling through Wnt5 or Wnt11 results in an increase in intracellular Ca^{2+} during zebrafish gastrulation. To determine if the Ca^{2+}/Calmodulin-dependent protein kinase, CaMK-II, is a potential downstream target of the Ca^{2+} increases during ncWnt activation, CaMK-II's role in C&E was assessed. This study identifies *camk2b1* and *camk2g1* as being necessary for C&E movements, and outlines the phenotype of the overall embryo as well as individual cells of *camk2b1* and *camk2g1* morphants. The defects of CaMK-II morphants are specifically linked to alterations in C&E cell movements, while cell fate and proliferation are unaffected. An increase in CaMK-II activation during gastrulation produces similar C&E defects, demonstrating the specificity of CaMK-II's activation in facilitating these highly coordinated cellular movements. We show that CaMK-II is working downstream Wnt 11 and in parallel to JNK signaling during gastrulation C&E. Overall, these data identify CaMK-II as a required component of C&E movements during zebrafish development, downstream ncWnt signaling, and altering cell migration through changes in cell shape

Chapter 1: Introduction of Morphogenic Cell Migration and the Key Regulators

Morphogenic Cell Migration

Multicellular organisms are composed of many different cell types that become organized during development into distinct tissues and organs. Directed cell migration is a complex process that facilitates this morphogenesis. There are three crucial steps required for morphogenic cell migration: first a cell or a group of cells must become directionally oriented and extend projections (lamellopodia and filopodia) to become motile, next cells are guided toward their target sites and lastly they must stop migrating upon arrival at their required location (Aman and Piotrowski, 2009). Each step of motility requires a set of highly coordinated protein interactions and activity levels, with the correct level of protein activation at the right time and place necessary for properly facilitating migration. Understanding the regulatory elements responsible for each step of cell migration is an ongoing project of the developmental biology field; with a complete understanding of the process and components still unclear.

During morphogenesis, cells can migrate as epithelial sheets with limited change in neighbor relationships or as individual cells migrating actively in large cohorts of closely interacting cells over long distances (Keller, 2005). Gastrulation is a period during the development of higher organisms when individual cells move extensively in a collective manner. Specifically, epithelial precursors of the mesendoderm undergo a partial (Xenopus, fish) or complete (chick, mouse) epithelial-mesenchymal transition (EMT) as they ingress through the blastopore or the primitive streak, respectively (Keller, 2005; Shook and Keller, 2003). Following ingression, cells continue to migrate leading to the formation of the embryonic axis. Understanding the mechanisms that guide this collective

cell migration and the mechanisms that execute it are of crucial importance in studying the development of higher organisms. This project aims to further elucidate these necessary cues using zebrafish as a model organism.

Early Zebrafish Development

Zebrafish (*Danio rerio)* have become a favorite model organism for studying embryonic development due to their small size and ease of culture. The development of zebrafish is similar to embryogenesis of higher vertebrates, including humans, with the exception of external fertilization and transparent eggs, which allow for easy observation of all developing stages. During development, the zebrafish embryo starts as a single blastomere sitting on top of a large yolk cell. Prior to the morphogenic processes of gastrulation, successive cell divisions must occur to form a large multicellular blastoderm. It is not until the 10^{th} cell division cycle that the embryo reaches the midblastula transition (MBT) which is characterized by a lengthening in the cell cycle, loss of cell synchrony, activation of transcription and the appearance of cell motility (Kane and Kimmel, 1993). At MBT, an epithelial layer known as the enveloping layer (EVL) forms, covering the underlying blastomeres. It is the job of the EVL to migrate all the way around the yolk cell, completely surrounding the yolk by the end of gastrulation and eventually forming the periderm of the zebrafish. During EVL formation, a multinucleated yolk syncytial layer (YSL) is also forming. This is a cortically located ring at the interface of the yolk and blastomeres, which forms when marginally located cells fuse to the yolk cell. The EVL and YSL interact extensively to facilitate the migration of the EVL, which moves as a collective sheet protecting the underlying blastomeres.

Epiboly is the first dedicated and collective cell movement of the internal blastomeres, where they begin to thin and spread around the underlying yolk cell (Fig. 1-1). These cells will continue to spread until the entire yolk cell is covered. In addition to cells moving in a vegetal direction to cover the yolk, interior cells of the blastoderm are also radially intercalating. These movements are thought to assist in epiboly and occur when more interior blastoderm cells move toward the external layers, facilitating the thinning process. The most superficial layer of cells, EVL, does not cooperate in these cell intercalations and instead continues to spread and cover the yolk, with cells at the leading edge narrowing and aligning to facilitate the yolk plug closure at the end of gastrulation (Keller and Trinkaus, 1987; Köppen et al., 2006). In addition to radial intercalations, the interaction of the EVL with the YSL also plays an important role in the epiboly of the EVL and underlying blastomeres. The YSL, EVL and the blastoderm interact with one another via microtubule and actin cytoskeleton structures and it is forces from these interactions that are believed to drive the migration of epiboly. At the onset of epiboly, the YSL is observed to contract and as epiboly continues, the animal-vegetal microtubules become shorter. In addition, an accumulation of actin in a ring-like band at the equator of the yolk cell occurs at 50% epiboly. Research has shown that this actin band is extremely important in the continued epiboly of both the EVL and blastoderm, with disruption resulting in epiboly defects of both layers (Cheng et al., 2004; Köppen et al., 2006; Zalik et al., 1999).

Internalization occurs after an embryo has reached 50% epiboly, and is defined as the morphological process resulting in the differentiation of the hypoblast (mesoderm and endoderm) (Fig. 1-1). Internalization occurs when marginal cells accumulate, forming what is known as the 'germ ring'. From here, the cells within the germ ring internalize and

begin to migrate toward the animal pole, while the overlying EVL and epiblast continue to migrate in the vegetal direction. The internalized cells consists of the endoderm, which will give rise to the epithelial lining of the digestive tract and associated glands, and is surrounded by the mesoderm which gives rise to muscles, skeleton and vasculature (Warga and Kimmel, 1990; Weijer, 2009). The mesoderm is surrounded by an outermost, non-involuting, epiblast (ectoderm), which will form the epidermis and the nervous system (Warga and Kimmel, 1990; Weijer, 2009).

At the onset of internalization, convergent extension (C&E) is simultaneously occurring where hypoblast and epiblast cells converge toward the future dorsal side of the embryo and upon arrival, extend in the anterior-posterior direction (Fig. 1-1). The dorsal side of the embryo is first evident when cells compact at one concentrated area and give rise to the shield, the embryonic organizer of the zebrafish (Warga and Nüsslein-volhard, 1998). The mesodermal cells located in the shield form axial structures such as the prechordal plate and notochord; whereas the surrounding paraxial and lateral mesoderm give rise to such structures as the somites and lateral plate, respectively (Warga and Kimmel, 1990). As gastrulation continues, cells of different mesodermal subtypes do not mix, and the degree of C&E movements for each subtype is based on their position along the dorsal-ventral axis (Glickman, 2003; Myers et al., 2002a; 2002b; Warga and Kimmel, 1990). Cells in the most ventral region experience no C&E, while cells of the lateral and dorsal regions experience increased levels of C&E as they move dorsally (Myers et al., 2002b; 2002a).

A major force driving C&E is mediolateral intercalation behavior (MIB) which occurs when axial mesodermal cells elongate along the mediolateral axis and use oriented bipolar

or monopolar protrusions to drive intercalation between their immediate neighbors (Wallingford et al., 2002). In addition to MIB, directed cell migration facilitated by extending cell protrusions is also a driving force of axis development (D'Amico and Cooper, 2001; Ulrich et al., 2003; 2005). Specifically, the prechordal plate progenitors, once internalized, undergo directed collective migration towards the animal pole, extending cell protrusions in the animal pole direction. MIB is not observed in these axial mesoderm cells, but rather the cells seem to use the overlying epiblast as a surface on which to migrate, thus travelling animally on a substrate that is moving vegetally (Montero et al., 2003).

Regulators of Cell Migration during Early Zebrafish Development

Each mode of cell migration during gastrulation has been thoroughly studied to determine the regulatory elements that facilitate their movements. Provided is an overview of each of their mechanisms.

Epiboly

As the first distinct cell movement of zebrafish development, epiboly requires the thinning and spreading of a multilayered cell sheet. The regulatory elements primarily responsible for epiboly are cell adhesions and cytoskeleton dynamics (Rohde and C.-P. Heisenberg, 2007; Lilianna Solnica-Krezel, 2006). Specific cell adhesions include: E-cadherin, epithelial cell adhesion molecule (EpCAM), prion, and protocadherins, which have all been shown to be necessary elements for proper epiboly with E-cadherin as the most thoroughly studied cell adhesion molecule (Málaga-Trillo et al., 2009; Slanchev et al., 2009; Wada et al., 2006). E-cadherin is detected at cell junctions throughout the embryo at blastula stage, with high

levels apparent in more superficial cells, including the EVL, and lower levels in deeper cells (Kane et al., 2005). A knockdown in E-cadherin causes inhibition of epiboly in the deep cells, while the superficial layers are able to recover and continue to move (Babb and Marrs, 2004).

Microtubules and actin cytoskeleton are also critical for normal epiboly (Lepage and Bruce, 2010). A dense meshwork of microtubules appears during the blastula stage, at the YSL, and extends towards the vegetal pole. The observation that these microtubules shorten over the course of epiboly suggests they may be facilitating the towing of the blastoderm in the vegetal direction (L L Solnica-Krezel and Driever, 1994). By high stage, filamentous actin (F-actin) forms a cortical belt around each cell of the EVL, in addition to two rings along the margin during late epiboly (Zalik et al., 1999). These cortical rings work together at the end of epiboly to facilitate a circumferential constriction at the margin, allowing for the closure the blastopore (Cheng et al., 2004).

Internalization

The formation of the hypoblast requires the internalization of mesoderm and endoderm precursor cells. Members of the Nodal family of TGFβ signals are essential inducers of mesoderm and endoderm cell fate in vertebrates; thus these signals are essential for the internalization cell movements of the prospective mesoderm and endoderm beneath the future ectoderm at the blastoderm margin during gastrulation (Schier, 2003). Nodal and Lefty, a Nodal antagonist and feedback inhibitor, work together so that only those cells expressing Nodal, the mesoderm and endoderm precursors, will involute while Lefty expressing cells, ectoderm precursors, continue in epiboly (Feldman et al., 1998; Gritsman

et al., 1999). Zebrafish embryos mutant for Nodal signaling completely lack the formation of the mesoderm and endoderm cells (Carmany-Rampey and Schier, 2001; Feldman et al., 2000). Conversely, overexpression of Nodal signaling or loss in Lefty results in fate transformation of ectoderm to either mesoderm or endoderm (Chen and Schier, 2001; Feldman et al., 1998).

Convergent & Extension

The third and final cell movement of gastrulation is convergent extension (C&E), where cells undergo mediolateral intercalations to narrow and extend the developing embryonic axis. Of the three cell movements that occur during vertebrate gastrulation, convergent extension is the most studied. Multiple mechanisms have been identified as regulators of these cell movements including the ncWnt pathway, PDGF-PI3K pathways, Jak/Stat, Slit/Robo, extracellular matrix and fibronectin, tissue interaction and differential adhesions (Rohde and C.-P. Heisenberg, 2007). While each of these regulators have been shown to play a role in facilitating C&E, the non-canonical Wnt (ncWnt) pathway is the most prevalent and will be the focus of this overview.

The Wnt family of glycoproteins is one of the most significant and actively studied groups of secreted extracellular molecules. Wnts bind 7-pass transmembrane receptors of the Frizzled (Fz) family to activate several intracellular signaling cascades known to regulate cell movement, polarity, as well as other developmental processes including fate determination and proliferation (Rohde and C.-P. Heisenberg, 2007). There are two primary branches of Wnt signaling, the canonical Wnt pathway and the non-canonical Wnt pathway. Activation of the canonical Wnt pathway leads to an accumulation of β-catenin in

the cytosol and subsequent translocation to the nucleus where it helps in the activation β-catenin dependent gene transcription. During zebrafish development, canonical Wnt signaling has primarily been linked to establishing cell fate (Hikasa and Sokol, 2013). The second branch of Wnt signaling is known as the non-canonical Wnt (ncWnt) pathway and within the ncWnt pathway there are two sub-branches: the Wnt/Planar Cell Polarity (PCP) pathway and the Wnt/Ca^{2+} pathway. Cell polarity and motility during zebrafish development are primarily facilitated through ncWnt signaling (C. Heisenberg et al., 2000; C. P. Heisenberg and Nüsslein-Volhard, 1997; Ulrich et al., 2005; 2003).

The Wnt/PCP pathway was first discovered and named based on its role in establishing cell polarity within the epithelial plane of *Drosophila* tissues. There are numerous PCP components shared between vertebrates and *Drosophila*, however it is still unclear how these components act to regulate gastrulation cell movements. In *Drosophila*, asymmetric accumulation of Fz, Dishevelled (Dsh), and Diego at the distal cell membrane and Strabismus and Prickle on the proximal side facilitates hair growth on the distal cell membrane, with disruption resulting in randomized hair formation (H. H. Strutt and D. D. Strutt, 2005). In vertebrates, however, no obvious asymmetry has been reported during gastrulation despite Fz and other components localizing to the cell membrane (M. Park and Moon, 2002). While Wnt/PCP signaling does play a pivotal role in polarized cell movement during vertebrate gastrulation, it may not have the same instructive role as seen in *Drosophila*. For instance, in zebrafish *silberblick/wnt11* (*slb*) mutant embryos, migration of the prechordal plate progenitors towards the animal pole is slower, less persistent and often straying from the normal path with cells extending randomized pseudopod-like

processes as opposed to being directionally oriented (C. Heisenberg et al., 2000; C. P. Heisenberg et al., 1996; Ulrich et al., 2005; 2003). However, because the cells do eventually reach the animal pole, it's believed that Wnt11 might be facilitating overall movement through polarization, rather than acting as a major directional cue (Rohde and C.-P. Heisenberg, 2007).

In *Xenopus* the receptor for Wnt11 has been identified as Fz7, and it is believed to be the same in zebrafish (Djiane et al., 2000; Sumanas et al., 2002; Sumanas and Ekker, 2001). As a downstream target of Fz, Dishevelled (Dsh) activity is essential for Wnt/PCP signaling (C. Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000). While Dsh activation occurs downstream both canonical and ncWnt signaling; activated canonical Wnt signaling leads to cytosolic Dsh localization where as ncWnt activation results in membrane localization of Dsh (Veeman et al., 2003; Wallingford et al., 2000). Interestingly, in *Xenopus*, researchers have found that membrane localization of Dsh is crucial for the activation of downstream PCP targets, as well as C&E movements (Habas et al., 2003; T. J. T. Park et al., 2005).

The subcellular restriction (distal localization) of Fz/Dsh during polarization of *Drosophila* epithelia may also be important for Wnt/PCP regulation during gastrulation. While Wnt/Fz/Dsh do not localize to a distinct side of gastrulating zebrafish cells, they do show membrane localization and during membrane localization cell-cell adhesions seem to be enhanced (Witzel et al., 2006). This discovery suggests that Fz/Dsh activity and thus membrane localization may be targeted to different temporal and spatial areas of the gastrula to coordinate specific movements within groups of cells, i.e. prechordal plate, during gastrulation (C. Heisenberg et al., 2000; Rohde and C.-P. Heisenberg, 2007).

Two of the primary signaling cascades activated downstream of Wnt/PCP activation include the Rho GTPases; Rho and Rac. These two pathways work in parallel to one another, downstream Dsh activation. Rho GTPases are well known for their regulatory elements in cell polarization, cytoskeleton organization, cell adhesion and gene transcription (Hall, 2005; Montero and C.-P. Heisenberg, 2004). Rho has been identified as a mediator of vertebrate signaling, specifically in regulating myosin and the actin cytoskeleton. During zebrafish development, inhibition of Rho interferes with actin distribution of early blastomeres, while overexpression of Rho has been shown to rescue the C&E defects of embryos lacking Wnt11 or Wnt5 (Marlow et al., 2002; Zhu et al., 2006). Rac signaling involves the activation of Jun N-terminal Kinase (JNK), a known target of PCP signaling in vertebrates and *Drosophila* (Hammerschmidt et al., 1996; M. Park and Moon, 2002; D. I. Strutt et al., 1997; Weber et al., 2000; Yamanaka et al., 2002). Alterations in JNK activity during vertebrate development have been shown to lead to disrupted vertebrate gastrulation and polarized cell movements (Habas et al., 2003; G.-H. Kim and Han, 2005; Ren et al., 2006; Tahinci and Symes, 2003; Yamanaka et al., 2002).

While the downstream targets of Wnt/PCP signaling in vertebrates have been extensively studied with several targets identified, the downstream targets of Wnt/Ca^{2+} signaling are less known. Researchers have found that activation of ncWnt signaling leads to an increase in intracellular Ca^{2+}; with mutations in ncWnt genes, Wnt5 and Wnt11, resulting in a reduction of Ca^{2+} and/or C&E defects during zebrafish development (Hammerschmidt et al., 1996; C. Heisenberg et al., 2000; Kilian et al., 2003; Slusarski et al., 1997; Westfall et al., 2003). Wnt/Ca^{2+} signaling has previously been shown to regulate tissue separation during gastrulation and may be necessary for cell adhesiveness; an

important element of collective cell migration (Lilianna Solnica-Krezel, 2006; Winklbauer et al., 2001). Taken together, vertebrate gastrulation research has shown that activation of the ncWnt pathway provides both polarization of cells and cell adhesiveness via the Wnt/PCP and Wnt/Ca^{2+} pathways; and it is believed that these branches of the ncWnt pathway may be working together to regulate directed cell migration necessary for vertebrate axis formation.

CaMK-II and Cell Migration

Ca^{2+}/calmodulin-dependent protein kinase type II, CaMK-II, is a developmentally essential serine/threonine protein kinase. During embryonic development CaMK-II has been shown to have key roles in neuron development and function, cell proliferation and cell migration (Easley et al., 2006; 2008; Fink et al., 2003; Johnson et al., 2000; Kühl et al., 2000a; 2000b; Sheldahl, 2003; Tombes and Peppers, 1995; Wen et al., 2004; Zou and Cline, 1996). Of particular interest for this project is CaMK-II's regulatory role in cell migration. Previous research has found that CaMK-II is necessary for the attachment and motility of human mammary epithelial cells (HME), Chinese hamster ovary cells (CHO) and vascular smooth muscle cells (VSM) (Bouvard and Block, 1998; Bouvard et al., 1998; Pauly et al., 1995; Pfleiderer et al., 2004). In addition, catalytically active CaMK-II has been shown to promote focal adhesion turnover necessary for the migration of mouse epithelial cells (Easley et al., 2008). In addressing vertebrate development, and specifically zebrafish, very little research has looked at CaMK-II's role during cell migration of gastrulation. Of the experiments performed thus far, CaMK-II activity has been linked to the ncWnt pathway. As previously noted, ncWnt signaling leads to an increase in intracellular Ca^{2+} during

gastrulation (Slusarski et al., 1997; Westfall et al., 2003); and zebrafish embryos mutant for ncWnts show a reduction in Ca^{2+} and/or C&E defects (Hammerschmidt et al., 1996; C. Heisenberg et al., 2000; Kilian et al., 2003). In *Xenopus* embryos, overexpression of Wnt5a mRNA results in an increase in Ca^{2+} and CaMK-II activity (Kohn and Moon, 2005) and in zebrafish, Wnt5 loss-of-function embryos can be partially rescued by overexpression of a truncated constitutively active CaMK-II construct (Westfall, 2003). The current research suggests that CaMK-II is acting downstream of ncWnt signaling during gastrulation and may be regulating C&E movements, but continued research is needed to confirm these ideas.

Importance of Cell Migration

Cell migration is a biological function that is required from conception to death and is primarily responsible for fulfilling such major biological functions as embryonic development and immunity. When cell migration is misregulated and/or improperly functioning it can have detrimental affects on an organism, including congenital birth defects and cancer. By understanding how cells move throughout a system normally, biologists will be able to better address the causes of defective cell migration in hopes of developing better targeted treatments.

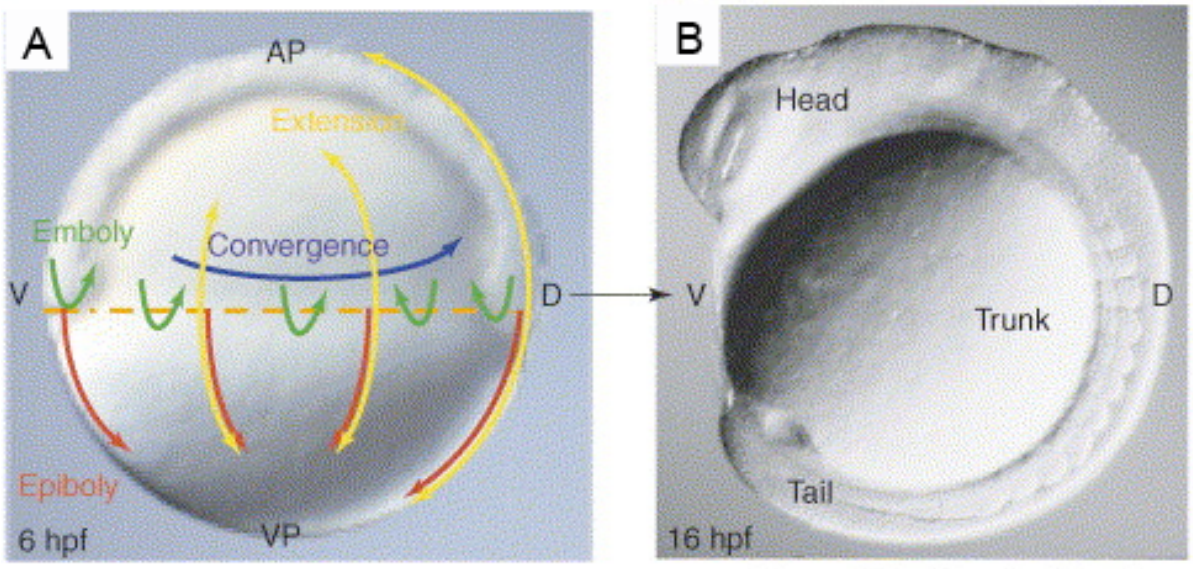
As extensively discussed thus far, cell migration is absolutely required for the overall development of an organism. Defects in migration at all stages of development have been shown to lead to severe embryonic malformations and result in drastic consequences ranging from early embryonic lethality to birth defects, and account for multiple human syndromes including neurological disorders, congenital heart disease, as well as physical

and mental retardation (Kurosaka and Kashina, 2008). Despite the differences in the cell types that take part in different migratory events, it is believed that all of these migrations occur by similar molecular mechanisms, whose major components have been functionally conserved in evolution (Franz et al., 2002). By increasing the molecular understanding of these migratory mechanisms, a means for the development of new therapies targeted towards these diseases in humans could be provided.

In multicellular organisms, most cells are confined to a particular tissue or organ via biological regulators such as cell-cell recognition and the basal lamina, which underlies most epithelial sheets and muscles and acts as a barrier for a particular tissue. During cancer, cancerous cells are able to invade different tissues; often causing devastating affects to the normal functions of that tissue. Understanding the invasion process of cancer is of great importance to the cancer research community, and a large part of the research efforts are currently focused on identifying the regulatory mechanisms of invasion. Interestingly, in studying cell migration researchers have found that tissue invasion is not always bad; in particular, researchers have found that during development there are also 'normal' tissue invasions with examples including border cell migration in the ovary of *Drosophila*, white blood cell emigration during inflammatory response, neural crest cell migration, and epithelial-mesenchymal interactions (Podbilewicz, 2003). By studying the regulatory mechanisms necessary during 'good' invasion, biologists hope to unlock the tricks of 'bad' invaders in addition to being able to specifically target these cells in cancer treatment.

Project Overview

The goal of this project is to identify CaMK-II as a significant molecular regulator of cell migration during development. Specifically, this projects aims to uncover CaMK-II's role in the convergent extension movements of zebrafish development. In addressing CaMK-II's function in migration during early development, key regulators of migration will be assessed including the cytoskeleton cellular network that facilitates movement as well as changes in cell shape within the different cellular layers. Finally, this project aims to identify CaMK-II as a downstream target of ncWnt signaling during gastrulation, while suggesting possible links CaMK-II may have with additional downstream ncWnt parallel pathways.



(Lilianna Solnica-Krezel, 2006)

Figure 1-1. Epiboly, emboly (Internalization), convergence and extension cell movements at shield stage lead to zebrafish axis formation. (A) Shield stage zebrafish embryo shows epiboly (red), emboly/internalization (green), convergence (blue) and extension (yellow) cell movements to facilitate the formation of the zebrafish body plan. (B) Head, trunk and tail formation by 16hpf, due to cell migrations occurring at 6hpf.

Chapter 2: camk2b1 and camk2g1 Mediate Proper Zebrafish Convergent Extension

Introduction

Convergent extension (C&E) is a key process by which tissues undergo narrowing along one axis and concomitant extension along another axis (Tada and C.-P. Heisenberg, 2012). The most prominently studied example of C&E is the development of the body axis during gastrulation (Keller et al., 2000). At the onset of gastrulation the three germ cell layers are specified; ectoderm, mesoderm and endoderm, and by the end of gastrulation these layers need to reorganize to form the embryo proper. Convergence of cells narrows the germ layers and the embryonic body mediolaterally, while extension movement elongates the embryonic tissue from head to tail (Lila Solnica-Krezel and Sepich, 2012).

At mid-gastrulation (7.5hpf), all three germ layers undergo C&E movements. While the C&E pattern of the endoderm and ectoderm is less understood, four distinct C&E zones have been identified in the mesoderm along the dorsal-ventral dimension of the zebrafish gastrula (Myers et al., 2002b; 2002a). The far ventral region of the embryo is defined as the 'no convergence, no extension zone' and cells in this region only move down the yolk and into the yolk bud where they will eventually form the embryonic tail (Myers et al., 2002a). Moving across the embryo in the dorsal direction, the next zone is the lateral domain where C&E movements are initially slow and accelerate as the cells move closer to the dorsal midline (Jessen et al., 2002; Myers et al., 2002b). The third C&E zone, the medial part of the mesoderm located approximately six-cell diameters to the axial mesoderm exhibits modest to high C&E rates (Glickman, 2003; Yin et al., 2008). Finally, in the most dorsal region of the embryo, axial mesoderm, cells show the same level of convergence as the medial mesoderm, with three-fold higher extension rates to facilitate the extension of the anterior-

posterior body axis (Gong and Korzh, 2006; Roszko et al., 2009; Lila Solnica-Krezel and Sepich, 2012). Extension is achieved via directed cell motility and mediolateral cell intercalation. To summarize, normal axis formation of zebrafish requires the combination of epiboly movements of ventrally located cells, dorsal migration of mediolateral cells and intercalation of midline axial cells.

The regulatory mechanisms that control C&E cell movements during zebrafish gastrulation have been extensively studied, with the ncWnt pathway identified as a crucial mediator (Seifert and Mlodzik, 2007). Wnt11 and Wnt5 have been identified as necessary ligands for normal cell movements during vertebrate C&E. The zebrafish mutant silberblick (*slb*) has a mutation in *wnt11*. In these mutants C&E movements of anterior and posterior mesendoderm and neuroectoderm is reduced leading to a shortened body axis at the end of gastrulation (C. Heisenberg et al., 2000; C. P. Heisenberg et al., 1996; C. P. Heisenberg and Nüsslein-Volhard, 1997). Using zebrafish pipetail (*ppt*) mutants, which contain a mutation in the *wnt5* gene, researchers found that Wnt5 regulates cell elongation and C&E in posterior mesendoderm and ectoderm regions of the gastrula, specifically regulating cell elongation and C&E movements (Kilian et al., 2003). *wnt5* was also shown to regulate cells in the anterior region of the zebrafish embryo however these affects are largely redundant to that of the *slb/wnt11* mutants (Kilian et al., 2003). Taken together these studies identified the ncWnt pathway as the crucial regulator of C&E, with defects in C&E characterized as a shortened body axis by the end of gastrulation, specifically reducing mesendoderm and neuroectorderm migration in the anterior and posterior regions of the developing embryo.

The protein of interest for this study is CaMK-II, the type II multifunctional Ca^{2+}/CaM-dependent protein kinase. CaMK-II is best known, during vertebrate development, as a downstream target of the ncWnt pathway. In *Xenopus,* CaMK-II is activated downstream Wnt5A or Wnt11 ligand binding is coupled with an increase in Ca^{2+} and is specific to the ventral side of the developing embryo where it is responsible for inducing ventral cell fate (Kühl et al., 2000a). In zebrafish, as noted earlier, the ncWnt pathway is known to regulate C&E rather than cell fate. Researchers have found that ectopic expression on ncWnt ligands enhances Ca^{2+} transients (Slusarski et al., 1997). In addition, C&E defects caused by a knockdown in ncWnt, Wnt5, can be partially rescued by the overexpression of CaMK-II (Westfall, 2003). The goal of this project is to identify if CaMK-II is necessary for zebrafish C&E movements during gastrulation.

This study characterizes CaMK-II's role during zebrafish gastrulation, identifying specific CaMK-II genes as being necessary for C&E movements and outlines the defects associated with a knockdown in CaMK-II during development. The defects of CaMK-II morphants are specifically linked to alterations in C&E cell movements with cell fate and proliferation unaffected. An increase in CaMK-II activation during gastrulation shows similar C&E defects as a knockdown in CaMK-II, demonstrating the specificity of CaMK-II's activation in facilitating these highly coordinated cellular movements. Lastly, we show that overexpression of dominant negative CaMK-II results in a range of C&E defects. Overall, this data identifies CaMK-II as a required component of C&E movements during zebrafish development.

Methods

Zebrafish strains and handling

Animal care and husbandry was performed according to methods previously described (Kimmel et al., 1995). Wild-type (AB and WIK) embryos were raised at 28.5°C and fed twice daily dry fish food, pellets or Tetra flakes, in combination with live brine shrimp. Zebrafish were reared on a 14:10h light:dark cycle. Water quality was checked during feedings to monitor temperature (25-31°C), conductivity (300-1500μS), and pH (6-8). Ammonia (0ppm(mg/L)), nitrite (0ppm (mg/L)), and nitrate (below 40ppm (mg/L)) levels of the water were measured on a bimonthly basis. The numbers listed represent the desired ranges for each condition. Wild-type zebrafish were bred by natural pairings in breeding tanks and the eggs laid were collected, transferred to embryo water and raised (Kimmel et al., 1995). Microinjections were performed at the one cell stage. All experimental embryos were sacrificed by 48hpf via prolonged immersion in tricane methanesulfonate (MS222, Sigma) 200mg/L, as outlined in the NIH guidelines for use of zebrafish.

Microinjections

Morpholino anti-sense oligonucleotides (MO's) were diluted in Danieau buffer (17 mM NaCl, 2 mM KCl, 0.12 mM MgSO4, 1.8 mM $Ca(NO_3)_2$, 1.5 mM HEPES, pH 7.6) and injected at the 1 to 4-cell stage (Westerfield, 1993). *camk2b1* and *camk2g1* MO's were designed by Gene Tools (Philomath OR) and injected at up to 4ng per embryo. The *camk2b1* MO complements mRNA sequence from -19 to +6 relative to the start codon (GGCCATGTCTTCCCGTCTCGGACTC). The camk2g1 MO complements mRNA sequence from

-13 to +12 relative to the start codon (AATTGTAGCCATGGTTGTGTGTGCGT). Morpholino stocks (1mM) were stored at -80°C. Prior to injection, MO aliquots were heated to 65°C for 5 minutes, cooled to room temperature and diluted in Danieau buffer (Westerfield, 1993). Zebrafish dominant negative ($K^{43}A$) GFP-tagged CaMK-II was inserted into the pFRM2.1 vector, which is under the control of the carp β-actin promoter (Eckfeldt et al., 2005) and is therefore universally expressed. GFP $K^{43}A$ CaMK-II cDNA was injected at the one-cell stage. All injections were performed at a constant injection volume of ~1 nl per embryo, volume analysis was performed for each injection via injection into mineral oil on a micrometer.

Whole mount in situ hybridization

Digoxigenin-labeled anti-sense riboprobes (0.5-1.5kb) were synthesized using T3 or T7 RNA polymerase from cloned cDNAs, hybridized with fixed embryos and then developed using alkaline phosphatase-conjugated anti-digoxigenin antibodies as described (Rothschild et al., 2007). The *camk2b1* and *camk2g1* probes were synthesized from partial zebrafish cDNA clones in the TOPO/TA vector, which had been amplified by RT-PCR (Rothschild et al., 2007). The *myoD* probe was synthesized from cDNA provided by James Lister, PhD; *ntl* and *bmp4* probes were synthesized from cDNA provided by Debbie Garity, PhD. The laboratory of Bernard and Christine Thisse, at the University of Virginia, performed the combination of *dlx3*, *hgg1* and *ntl* staining using their probes.

RT-PCR

Total RNA was prepared from dechorinonated embryos at 75% epiboly and cDNA was prepared as described (Lister et al., 2001). Redundant and gene-specific PCR primers, which flank the variable region of each gene, were used to amplify oligo dT-primed cDNA, synthesized from total RNA prepared. PCR products were cloned and screened by sequence (Rothschild et al., 2007).

Immunolocalization

Embryos were fixed in fresh 4% paraformaldehyde for 2-4hrs at room temperature, washed in PBT (1xPBS, tween-20) and incubated in primary antibody. Primary antibodies included rabbit polyclonal antibody against activated Caspase 3 (Cell Signaling Technologies: Beverly, MA), rabbit polyclonal antibody against phosphohistone H3 (Biotech Assays). Goat anti-rabbit Alexa^{488} secondary was used after all primary antibodies. Caspase 3 and Phospho-histone H3 antibody stained embryos were counter stained with propidium iodide (Sigma).

Thapsigargin Treatment

The ER Ca^{2+} pump was suppressed with 0.25-1μM thapsigargin (Sigma), added to the culture medium from a 100x stock diluted in dimethyl sulfoxide (DMSO). As a control, DMSO was diluted 100x in culture medium. Embryos were treated from 5 to 7hpf, washed, and raised inside their chorions until the desired stage of development.

Microscopy

Live embryos were imaged using differential interference contrast optics after transient anesthesia with 0.003% Tricane (MS222, Sigma) and immobilization between coverslips. NIKON 20X, 40X and 100x oil immersion Plan APO objectives were used for confocal imaging.

Measurements and Statistical Analysis

Angle and length measurements were collected using Nikon Elements software. Cell count data was standardized by counting cells within a 161.5x161.5μM box drawn over the lateral region of the embryo using Nikon Elements software. Statistical analysis of body gap angle measurements, length:width ratios and cell count data was performed using Microsoft Excel t-test functions. All t-tests were 2-tailed, unpaired, and assuming unequal variance. An asterisk denotes statistically significant differences with *P*-values indicated in figure legends.

Results

Knockdown of camk2b1 and camk2g1 results in somite compression, tail curvature and truncation

Seven transcriptionally active genes encoding CaMK-II have been identified and described in early zebrafish embryos (Rothschild et al., 2007). Of these genes, *camk2b1* and *camk2g1* were investigated for their role in the development of the embryonic body axis. Gene specific anti-sense morpholino oligonucleotides (MO's) against *camk2b1* and *camk2g1* mRNA were injected into wild-type zebrafish embryos at the 1-cell stage. The embryos were raised to 24hpf and examined for potential defects. Both CaMK-II morphants

appeared abnormal in their overall body development. Wild-type embryos exhibited elongation of the tail and yolk extension (denoted by an asterisk), with clearly developed and chevron shaped somites (denoted by an arrow) (Fig. 2-1A). *camk2b1* morphants exhibited somite compression, notochord undulation with curvature of the tail and truncation of the yolk extension (Fig. 2-1B). *camk2g1* morphants displayed severe somite compression with truncation of the tail and yolk extension (Fig. 2-1C). As noted by the group morphant images, phenotypes from each experimental condition were reproducible and consistent (Fig. 2-1A',B'&C')

The hallmark features of an embryo with disrupted C&E include a shorter and broader body axis with defects in elongation during segmentation leading to undulation of the notochord (Hammerschmidt et al., 1996). The 24hpf phenotype presented by *camk2b1* and *camk2g1* morphants strongly suggest that C&E is affected when these CaMK-II genes are knocked down. Further experimentation would need to be performed in order to confirm our hypothesis. Note that no phenotype was detected when *camk2b1* and *camk2g1* morpholino co-injections were performed at concentrations lower than those used to cause C&E defects alone (data not shown).

camk2b1 and camk2g1 expression during early zebrafish development

Having found that a knockdown in either *camk2b1* or *camk2g1* produces a significant body axis formation defect commonly associated with defects in gastrulation convergent extension (C&E), the expression of these two genes was verified during the affected time points. C&E cell movements are most prevalent at 75% epiboly, mid-gastrulation, during zebrafish development (Yin et al., 2009). RNA from wild-type embryos at 75% epiboly

(~8hpf) was reverse transcribed and the subsequent cDNA was used in polymerase chain reactions (PCR) with *camk2b1* and *camk2g1* gene specific primers to assess if the genes were present. Gel electrophoresis confirmed that both *camk2b1* and *camk2g1* are present at 75% epiboly (Fig. 2-2A&B).

To determine localization of *camk2b1* and *camk2g1* expression, wild-type embryos were assessed by whole mount *in situ* hybridization using mRNA probes for *camk2b1* and *camk2g1*. Embryos were fixed at several time points before, during, and after zebrafish gastrulation to visualize if expression fluctuates during early development. *camk2b1* expression was faint at sphere stage, with little to no expression at shield stage (Fig.2-2C&D). By 75% epiboly, expression was detected within the anterior region of the developing embryo, which continued at bud stage (arrows) (Fig.2-2E&F). By 24hpf *camk2b1* was globally expressed in the zebrafish embryos, showing strong expression in the head and throughout the trunk and tail (Fig.2-2G).

Expression of *camk2g1* was detected throughout early zebrafish development including blastula, gastrula and 24hpf stages. During sphere stage *camk2g1* expression was faint but present and by shield stage expression was clearly detected in all areas of the blastoderm at equal levels with no sidedness (Fig.2-2H&I). By 75% epiboly and bud stage, expression was detected in both the anterior and posterior regions of the developing embryonic body (arrows)(Fig.2-2J&K). At 24hpf, global expression of *camk2g1* was detected, showing strong expression in the brain, trunk and tail regions (Fig. 2-2L).

CaMK-II morphants express increased gap angle measurements

A classic characteristic of disrupted C&E during zebrafish gastrulation is truncation of the anterior-posterior (A-P) body axis (Hammerschmidt et al., 1996; C. Heisenberg et al., 2000). Although the 24hpf phenotype of *camk2b1* and *camk2g1* morphants show trunk extension defects, quantification of this phenotype was necessary. To quantify the truncation of the A-P body axis, gap angle measurements of 10±1 somite stage (10±1ss) embryos were assessed. The gap angle is defined as the angle formed between the head, mid-yolk and tail of laterally positioned embryos at the 10±1ss of zebrafish embryos (denoted by dashed lines) (Fig. 2-3).

camk2b1 and *camk2g1* morphants produced similar gap angle measurements, with an increase in the average angle for *camk2b1* MO's from 29° (control) to >69° (Fig. 2-3B&D). *camkg1* morphants showed a slightly more severe phenotype, with an increase in the average gap angle from 29° (control) to >75° (Fig. 2-3C&D). The increases in 10±1ss gap angles for both morphants are statistically significant with $P<0.001$ (asterisk) (Fig.2-3D).

camk2b1 and camk2g1 morphants display C&E defects of the hypoblast

C&E cell movements primarily occur in the anterior and posterior mesendoderm and neuroectoderm cell layers during gastrulation (Copp et al., 2003; C. Heisenberg et al., 2000; C. P. Heisenberg et al., 1996; C. P. Heisenberg and Nüsslein-Volhard, 1997; Kilian et al., 2003). Mesendoderm tissue is defined as the hypoblast during zebrafish gastrulation and is composed of axial and paraxial cells (Westerfield, 1993). To determine if a knockdown in CaMK-II is altering C&E of the hypoblast, *in situ* hybridization was performed on morphant embryos using the axial and paraxial riboprobes *ntl* and *myoD,* respectively.

Two time points were chosen for hypoblast analysis, 90% epiboly and early somitogenesis (6-7somites). At 90% epiboly the axial tissue (*ntl)* appears wider in *camk2b1* morphants, with wider and shorter expression in *camk2g1* morphants when compared to control embryos (Fig.2-4A,B&C). *MyoD* staining in 90% epiboly embryos shows mislocalization in morphant embryos. Control embryos express *myoD* in the posterior region of the embryo, with tight parallel expression at the base of the developing notochord (arrow) (Fig.2-4A). In *camk2b1* morphants the expression of *myoD* appears widened and less organized, whereas in *camk2g1* morphants *myoD* expression has not migrated from the germ ring (Fig.2-4B&C). These results indicate *camk2b1* and *camk2g1* morphants express defects of mesendoderm migration by the end of gastrulation.

To determine if the developmental recover later in gastrulation, *myoD* and *ntl* expression was also assessed at the 6-7somite stage (ss). Control embryos show chevron shaped and clearly separated somites (Fig. 2-4D). *camk2g1* morphants display a complete loss in the chevron shape of somites, and instead appear completely horizontal with a decrease in somite separation (Fig.2-4F). *camk2b1* morphants appear to experience an intermediate phenotype, with the somites neither chevron shaped nor completely horizontal (Fig.2-4E).

L:W ratio data were collected using *myoD* and *ntl* stained zebrafish embryos ranging from the 6-10ss. Length measurements were collected starting at the most anterior region of the first somite and extending to the posterior region of the sixth somite, with width measurements taken from the sixth somite (denoted by solid and dashed lines, respectively) (Fig.2-4D). The sixth somite was chosen for width measurements to ensure uniformity, rather than measuring from the widest somite for each measurement. *camk2b1*

and *camk2g1* morphants produced similar decreases in L:W ratio measurements, with a decrease in the average ratio for *camk2b1* morphants from 1.37±0.151 (control) to 1.17±0.079 (Fig. 2-4D,E&G). *camkg1* morphants showed a slightly more severe phenotype, with a decrease in the average L:W ratio from 1.37±10.151 (control) to 0.899±0.17 (Fig. 2-4D,F&G). The decrease in L:W ratio measurement for both morphants is statistically significant with $P<0.001$ (asterisk) (Fig.2-4G). Based on the disrupted axial and paraxial markers of late gastrulation and the L:W ratios of early somitogenesis embryos, these data confirm that C&E of the hypoblast in CaMK-II morphants is defective by late gastrulation and the problem continues through somitogenesis.

camk2b1 and camk2g1 morphants display neuroectoderm C&E defects

As noted previously, C&E cell movements occur in the mesendoderm and neuroectoderm embryonic layers during zebrafish development. C&E of the neuroectoderm is required to narrow and elongate the neural floor plate, which then folds appositionally to form the neural tube (Copp et al., 2003). To determine if a knockdown in CaMK-II is altering neuroectoderm C&E, *in situ* hybridization was performed on morphants using the mRNA probes *dlx3, hgg1* and *ntl*. By marking the neural plate (*dlx3*), hatching gland/prechordal plate (*hgg1*) and notochord (*ntl*) of 1-3 somite embryos; C&E defects were assessed in CaMK-II morphants. The marking of the neural plate allowed for the assessment of neuroectoderm C&E, while the *hgg1* and *ntl* probes in the same embryos were able to confirm anterior prechordal plate and axial C&E alterations, respectively.

The neural plate boundary, marked by *dlx3,* completely surrounds the developing zebrafish embryo by early somitogenesis and is responsible for patterning the midbrain

and hindbrain primordial of the neural plate later in development (Westerfield, 1993)(Fig. 2-5A). The neural plate converges anteriorly by 1-3 somites and the polster of cells comprising the hatching gland/prechordal plate (*hgg1)* are positioned directly anterior the tip of the converged neural plate boundary (Fig.2-5A). Moving in the posterior direction, the neural boundary widens slightly, running parallel to the notochord (*ntl)* (arrows)(Fig.2-5D). *camk2b1* and *cam2g1* morphants both express similar defects in neural plate boundary formation. In the morphants, the anterior neural plate boundary does not converge in the anterior region, and is greatly widened when compared to control embryos (Fig.2-5B&C). This widened neural plate expression continues into the posterior region of the embryo, so much so that the expression of *dlx3* in the posterior view is only noticeable at the far lateral edges of the embryo (arrows) (Fig.2-5E&F).

In addition to the widened neural plate boundary, CaMK-II morphants also show a defect in the anterior migration of the prechordal plate (*hgg1*) and convergence of the notochord (*ntl*). *camk2b1* and *cam2g1* morphants display *hgg1* expression posterior to the neural plate boundary (*dlx3*), confirming anterior migration is defective (Fig.2-5B&C). In addition, morphant *ntl* expression confirms a widening of the axial tissue (Fig.2-5E&F). These results show that a knockdown in CaMK-II results in defective neuroectoderm C&E, and confirms previous results of defective C&E in the anterior hypoblast and axial mesoderm.

Cell fate specification is unaltered in CaMK-II morphants

The reduced convergence and extension of axial, paraxial and neuroectoderm tissue observed in CaMK-II morphants suggests impairment of C&E movements during

gastrulation. However, these results could also be explained by alterations in cell fate specification. These two processes, although very different, occur at the same time during development and produce similar phenotypes when altered. To test whether cell fate specification was affected, whole mount *in situ* hybridization was performed using the ventral fate marker *bone morphogenetic protein 4* (*bmp4)*.

During zebrafish gastrulation, a ventral to dorsal gradient of bone morphogenetic (Bmp) activity establishes cell fates (Myers et al., 2002a), with the highest concentration of Bmp localized to the most ventral region of the embryo and little to no expression at the dorsal region. To determine whether cell fate specification is altered in CaMK-II morphants, expression of *bmp4* was assessed at the onset of gastrulation (shield stage) and during mid-gastrulation (75% epiboly). The expression pattern of *bmp4* remained unchanged in both *camk2b1* and *camk2g1* morphants at shield stage and 75% epiboly when compared to wild-type siblings (control) (Fig.2-6).

Proliferating Cell Number is Unaltered in CaMK-II Morphants

CaMK-II is defined as a multifunctional protein kinase known to play a role in a variety of developmental processes aside from cell migration; including cell proliferation (Baitinger et al., 1990; Tombes et al., 1995; Tombes and Peppers, 1995). To determine if the proposed defective C&E phenotype experienced by CaMK-II morphants is actually caused by a change in cell proliferation and not cell movement, the percentage of proliferating cells in morphant embryos was assessed.

An antibody against phosphohistone-H3 at Serine-10 was used in combination with the vital dye propidium iodide to assess proliferating cell numbers in zebrafish embryos.

Histone H3 is a core histone protein and major constituent of chromatin. During mitosis, phosphorylation of serine-10 on histone H3 occurs to facilitate chromosome condensation necessary for cell division, thus the antibody can be used to identify newly dividing cells. Propidium iodide is an intercalating agent that binds to nucleic acids, thus staining the nucleus of all cells. To determine the percentage of proliferating cells in zebrafish embryos; the number of phosphohistone H3 positive cells was divided by the total number of cells counted within a 161.5 x161.5 μM box drawn within the middle region of the embryo.

Percentage of embryonic proliferating cells was assessed at the onset of gastrulation (shield stage) and during mid-gastrulation (75%). The percentage of proliferating cells within shield stage embryos was 7.14% (±0.034%) for control, 6.85% (±0.018) for *camk2b1* morphants and 6.89% (±0.019) for *camk2g1* morphants; counting cells from three embryos for each condition (Fig. 2-7A,B,C&G). In assessing 75% epiboly embryos, control embryos displayed a proliferation rate of 7.1% (±0.2), *camk2b1* morphants 7.4% (±0.1) and *camk2g1* morphants 5.75% (±0.1), counting cells from three embryos for each condition (Fig. 2-7D,E,F&G). T-test statistical analysis found that the percentage of proliferating cells between the control and either CaMK-II morphant was not statistically significant (*P>0.1)*(Fig. 2-7G). To conclude, the number of proliferating cells in the beginning and mid-gastrulation stages are unchanged between control and CaMK-II morphant embryos.

Apoptotic Cell Number is Unaltered in CaMK-II Morphants

While a knockdown in CaMK-II does not have an affect on the rate of proliferation in zebrafish embryos prior to or during zebrafish gastrulation, alterations in apoptosis could

explain the phenotypic changes of CaMK-II morphants. A change in apoptotic cell number within a developing embryo could explain the phenotype of CaMK-II morphants rather than a defect in C&E cell movements. To determine the number of apoptotic cells within an embryo, an antibody against activated Caspase 3 (Cell Signaling) was used in combination with propidium iodide at shield and 75% epiboly stages of zebrafish development. Activated Caspase 3 is essential for the sequential activation of Caspases responsible for the execution of apoptosis, and thus serves as a great indicator for apoptotic positive cells.

Gastrulation stage zebrafish embryos, shield and 75% epiboly, show little to no apoptosis with approximately 0-5 cells expressing activated Caspase 3 (Fig.2-8A&D). Assessment of *camk2b1* and *camk2g1* morphants also shows that the number of cells positive for activated Caspase 3 is approximately 0-5 cells (Fig.2-8B,C,D&E); three embryos for each condition were assessed. No significant change in activated Caspase 3 positive cells was noted between control and CaMK-II morphants.

Overexpression of Dominant Negative CaMK-II displays a range of suppressed cell movement phenotypes

CaMK-II is a unique protein kinase in its ability to autophosphorylate adjacent subunits allowing for continued activation of the enzyme in the absence of Ca^{2+}/CaM (Lou and Schulman, 1989; S. G. Miller et al., 1988; Schworer et al., 1988; Thiel et al., 1988). The CaMK-II point mutagen $K^{43}A$, disrupts the catalytic domain of CaMK-II, rendering it incapable of autophosphorylation. By inhibiting the catalytic domain of one subunit of CaMK-II, autophosphorylation is blocked for the entire hetero-oligomerized CaMK-II structure (Johnson et al., 2000).

To determine how a knockdown of CaMK-II in a subset of cells will affect zebrafish development, GFP-tagged $K^{43}A$ CaMK-II cDNA was injected into zebrafish embryos at the one-cell stage. Unlike MO's, CaMK-II cDNA will not be incorporated into each cell and thus produces mosaic expression with only a subset of cells expressing the construct. Three concentrations of GFP $K^{43}A$ CaMK-II were injected (80, 100 and 120ng) to determine potential dose-dependent affects. Embryonic phenotype was assessed at 24hpf for all injected embryos. The results show that 35-60% of GFP $K^{43}A$ CaMK-II expressing embryos show normal development at all concentrations of injection (Fig. 2-9A&I). The compressed phenotype, previously noted as a C&E defect and expressed in *camk2b1* and *camk2g1* morphants, was seen in 25-35% of $K^{43}A$ expressing embryos (Fig. 2-9B&I). Interestingly, a new phenotype termed midline bifurcation (MB), never seen with CaMK-II morpholino injections, was identified when embryos were injected with the $K^{43}A$ CaMK-II construct. Within these embryos the posterior trunk/tail region of the embryo does not completely fuse and somites are seen on either side of a notochord with a portion of the yolk exposed in the middle (Fig. 2-9C'&D'). In MB embryos, high GFP $K^{43}A$ CaMK-II expression was seen in the cells around the bifurcation (arrow) (Fig. 2-9C). The bifurcation phenotype was seen in 16% of 80ng injected embryos, 24% of 100ng and 29% 120ng; showing a clear dose-dependent affect (Fig. 2-9I).

In situ hybridization using the axial and paraxial markers *ntl* and *myoD,* respectively, were used to further assess the midline bifurcation phenotype of GFP $K^{43}A$ CaMK-II embryos. Three representative images show that in each of the bifurcated embryos, somite expression is limited to one side of the notochord and no somites are seen within the interior region of the split (*ntl* denoted by asterisks; *myo*D denoted by arrows)(Fig. 2-9E-

H). Together, these results show a range in phenotypes during mosaic inhibition of CaMK-II including a new C&E defect, not seen with CaMK-II morphants, midline bifurcation.

Pharmacologically induced increases in intracellular Ca^{2+} during zebrafish gastrulation results in increased CaMK-II autonomy and C&E defects

Ca^{2+} signals have been proposed as major regulatory means for a variety of developmental events including fertilization, cell polarity, cell division, cell migration, dorsal-ventral axis patterning, brain and heart development (Parrington et al., 2007; Slusarski and Pelegri, 2007; S. E. Webb and A. L. Miller, 2006; Whitaker, 2006). Inhibition of the endoplasmic reticulum (ER) Ca^{2+} pump, via thapsigargin treatment, prior to and during zebrafish gastrulation has previously been shown to induce phenotypes similar to a loss in ncWnts (Creton, 2004). Overexpression of ncWnts during vertebrate development has also been shown to cause C&E defects similar to non-canonical Wnt suppression (Du et al., 1995; Moon et al., 1993). To determine if an increase in cytosolic Ca^{2+} during zebrafish gastrulation will cause an increase in CaMK-II activity and subsequent defective C&E, embryos were treated with thapsigargin.

Thapsigargin, a guaianolide compound that was isolated from the Mediterranean plant *Thapsia garganica*, inhibits the ER Ca^{2+} pump, flooding the cell with free cytosolic Ca^{2+} (Treiman et al., 1998). Zebrafish embryos were raised in regular fish water and treated with increasing concentrations of thapsigargin during time points consistent with the onset of gastrulation, 5-7hpf, with control embryos treated with 1% DMSO. Embryos were analyzed at the 10ss, 12-16ss and 24hpf.

To determine if an increase in cytosolic Ca^{2+} also results in an increase in CaMK-II activity, an autocamtide-2 peptide-based assay was performed on whole embryo lysates at the 12-16ss and 24hpf. The results of this assay show CaMK-II autonomy of 12-16ss embryos significantly increases in a dose-dependent manner and that this increase in autonomy subsided by 24hpf (Fig.2-10A).

Next, to determine if an increase in embryonic CaMK-II autonomy results in C&E defects similar to those seen during a knockdown of CaMK-II, gap angle measurements at 10±1ss embryos and 24hpf morphology were assessed. Control (DMSO) 10±1ss embryos exhibited a gap angle of 34.17°(±4.5), with all thapsigargin treated embryos resulting in statistically significant (*P<0.001) and dose-dependent increases in gap angles. 0.25μM thapsigargin treated embryos showed a gap angle of 87.43° (±21.56), 0.5μM 128.5° (±19.44) and 0.75μM 151.14° (±22.5) (Fig. 2-10B,D,F,G&J). Further assessments show 24hpf treated embryos with severe axis compression defects increasing in severity, with increasing concentrations of thapsigargin (Fig. 2-10C,E,G&I). These results show that embryos treated with thapsigargin during early zebrafish gastrulation experience an increase in CaMK-II activity, increase in body gap angles at 10±1ss and compression of the A-P body axis by 24hpf

Discussion

Gastrulation convergent extension (C&E) cell movements facilitate proper embryonic axis development. Proposed modulators of C&E movements have been extensively studied, with ncWnts identified as essential regulators for these cellular movements (Kühl et al.,

2000b; Markova and Lenne, 2012; Slusarski and Pelegri, 2007; Tada et al., 2002; Veeman et al., 2003; S. E. Webb and A. L. Miller, 2006). Over-expression or loss of non-canonical Wnt signaling results in C&E defects during zebrafish development (C. Heisenberg et al., 2000; Kilian et al., 2003). While it is clear that ncWnt proteins play an essential role in C&E; additional proteins and signaling pathways may also be involved. The results presented here show CaMK-II, a developmentally essential protein kinase, as a necessary protein for C&E. This work shows where in a developing embryo C&E movements are disrupted during CaMK-II inhibition, and demonstrates that these defects are in fact due to disrupted C&E and not alternate hypotheses of a change in cell fate, proliferation or apoptosis. Misregulated CaMK-II, whether through inhibition or over-expression, results in defective C&E movements. Together this work clearly demonstrates CaMK-II as a novel protein in regulating the C&E movements of vertebrate gastrulation.

Morphant analysis at 24hpf demonstrates that a knockdown in two of the seven zebrafish CaMK-II genes, *cam2b1* and *camk2g1*, disrupts the proper formation of the embryonic body axis (Fig. 2-1). CaMK-II morphants exhibit a similar phenotype to non-canonical Wnts, Wnt11 and Wnt5, morphant and mutant embryos (C. Heisenberg et al., 2000; Kilian et al., 2003; Lele et al., 2001). Defects include a shorter and wider body axis by 24hpf with undulation of the notochord (Hammerschmidt et al., 1996; C. Heisenberg et al., 2000; C. P. Heisenberg and Nüsslein-Volhard, 1997). The defects associated with a knockdown in ncWnts have been characterized as gastrulation C&E defects; thus determining if the defects seen by CaMK-II morphants are due to disrupted C&E was the next logical step.

Expression of CaMK-II was assessed for time points consistent with C&E cell movements. RT-PCR and *in situ* hybridization confirmed that *camk2b1* and *camk2g1* are expressed during gastrulation (Fig. 2-2). Gap angle measurements were used to quantitate the defects of *camk2b1* and *camk2g1* morphants and confirm that these defects directly correspond with disrupted C&E. Representative images and gap angle measurements clearly show that *camk2b1* and *camk2g1* morphants experience gap angle measurements that are double the average gap angle of control embryos (Fig. 2-3).

At the onset of gastrulation, differentiation of the three germ layers has occurred and cell movements are necessary to facilitate the organization and separation of the layers within a developing embryo. C&E cell movements are required within the mesendoderm and neuroectoderm layers to properly form the embryo. The neuroectoderm layer localizes to the most anterior region of the embryo, whereas the mesendoderm layers are predominantly localized in more posterior regions. To determine if CaMK-II is necessary for both anterior and posterior C&E during gastrulation, *in situ* hybridization for mesendoderm and neuroectoderm markers was assessed.

Mesendoderm mRNA probes *ntl* and *myoD* were used to asses axial and paraxial tissue, respectively, at late gastrulation and early somitogenesis time points. Length:width (L:W) measurements were used to quantitate the C&E of these tissues at early somitogenesis. CaMK-II morphants display significant disruption of mesendoderm by late gastrulation, which continues in early somitogenesis time points (Fig. 2-4).

Riboprobes *dlx3, hgg1* and *ntl* were used in combination to assess defects in the neuroectoderm and anterior/posterior embryonic formation. The neuroectoderm, neural plate, is marked by *dlx3*, which is clearly widened in both the anterior and posterior

regions of morphant 1-3 somite stage (1-3ss) embryos (Fig. 5B,C,E&F). In addition, the most anteriorly migrating cells, prechordal plate cells, identified by *hgg1* expression was found to be expressed posterior to the expression of *dlx3*, neural plate, indicative of a loss in anterior extension during gastrulation for both CaMK-II morphants. Finally, *ntl* expression of these morphant embryos confirms the widening of axial tissue. Taken together these results confirm the mesoderm C&E defects previously noted and show that neuroectoderm C&E is also altered in CaMK-II morphants.

While C&E is the most likely explanation for the defects seen during a knockdown of CaMK-II, alternate hypotheses are possible and thus needed to be tested. The first alternate hypothesis was that a knockdown in CaMK-II might alter cell fate specification. In altering the fate of the cells, the number of cells to form the dorsal, ventral, anterior and posterior region of the embryo would become disrupted and a 'dorsalized' or 'ventralized' phenotype, similar to the one seen by 24hpf CaMK-II morphants, would be expressed.

A gradient of bone morphogenic protein (*bmp*4) is responsible for establishing the ventral-to-dorsal fate of the embryo, with the highest expression of *bmp*4 specifying ventral fate and the least expression specifying the dorsal fate (Myers et al., 2002a). The *bmp4* expression gradient was assessed in CaMK-II morphants at shield and 75% epiboly. CaMK-II morphants display no change in the *bmp*4 expression gradient either at the beginning of gastrulation or during mid-gastrulation time points (Fig. 6). These results confirm ventral-dorsal cell fate induction occurs normally in CaMK-II morphants and supports the notion that altered cell fate does not account for the defects observed in morphants.

Additional alternate hypotheses to explain the phenotype of CaMK-II morphants are that CaMK-II may be altering the proliferative state of cells and/or degree of apoptosis. Antibody staining was used to determine the number of proliferative and apoptotic cells in CaMK-II morphants, and statistical analyses were performed. Antibody analysis found that a knockdown in either *camk2b1* or *camk2g1* had no affect on the number of proliferating or apoptotic cells at the beginning of gastrulation (shield stage) or mid-gastrulation (75% epiboly) (Fig. 2-7&8). Taken together, these results confirm that a loss in CaMK-II during zebrafish development is not altering the rate of proliferation or apoptotisis. In combination with the cell fate results, these results disprove all alternate hypotheses for the phenotype presented by CaMK-II morphants and further support CaMK-II's role as a regulator of C&E cell movements during zebrafish gastrulation.

To determine if mosaic inhibition of CaMK-II's would alter C&E, dominant negative CaMK-II cDNA was injected into the fish and embryos were examined at 24hpf. Dominant negative CaMK-II is mutated within the catalytic domain ($K^{43}A$) so that autophosphorylation cannot occur on adjacent subunits. Upon forming a dodecameric hetero-oligomerized structure with endogenous CaMK-II, the $K^{43}A$ CaMK-II construct will block autophosphorylation rendering the entire CaMK-II structure inactive. $K^{43}A$ cDNA will only become incorporated into a subset of cells, thus mosaic expression of the dominant negative CaMK-II construct was seen during this experiment. The results show that only a portion of embryos expressing dominant negative CaMK-II presented with C&E defects at 24hpf, with a large portion of embryos showing no defects (Fig. 2-9A,B&I). Interestingly, a new and more extreme defective C&E phenotype was noted in embryos where there was a cluster of $K^{43}A$ CaMK-II expressing cells within the mid-trunk region of the embryo. The

cells that form the mid-trunk region of the developing zebrafish embryo are primarily located within the lateral region of the embryo at the onset of gastrulation (50% epiboly) and thus require high levels of C&E in order to reach their final destination on the dorsal side of the embryo. When a cluster of cells within this region expressed dominant negative CaMK-II, wild-type cells were unable to rescue the migration defects and instead a midline bifurcation was formed (Fig.2-9). These results further confirm CaMK-II's requirement for C&E migration.

Previous research assessing Wnt's role in C&E during gastrulation found that both gain and loss of function manipulations impairs C&E cell movements (Matsui et al., 2005; Tada et al., 2002). To determine if the same is true for CaMK-II modulations, CaMK-II autonomy was increased during gastrulation and the 10ss and 24hpf phenotype of embryos was assessed. CaMK-II activity was increased during gastrulation through thapsigargin drug treatment between 5-7hpf (shield-70% epiboly). Quantitative assessment of CaMK-II activity and gap angle measurements at early somitogenesis stages found that thapsigargin treatment did result in an increase in CaMK-II activity and gap angle measurements in a dose-dependent fashion (Fig. 10A&C). These results further support CaMK-II's role in C&E; specifically showing that an increase in CaMK-II activity during gastrulation results in similar C&E defects as seen during a knockdown in *camk2b1* or *camk2g1.*

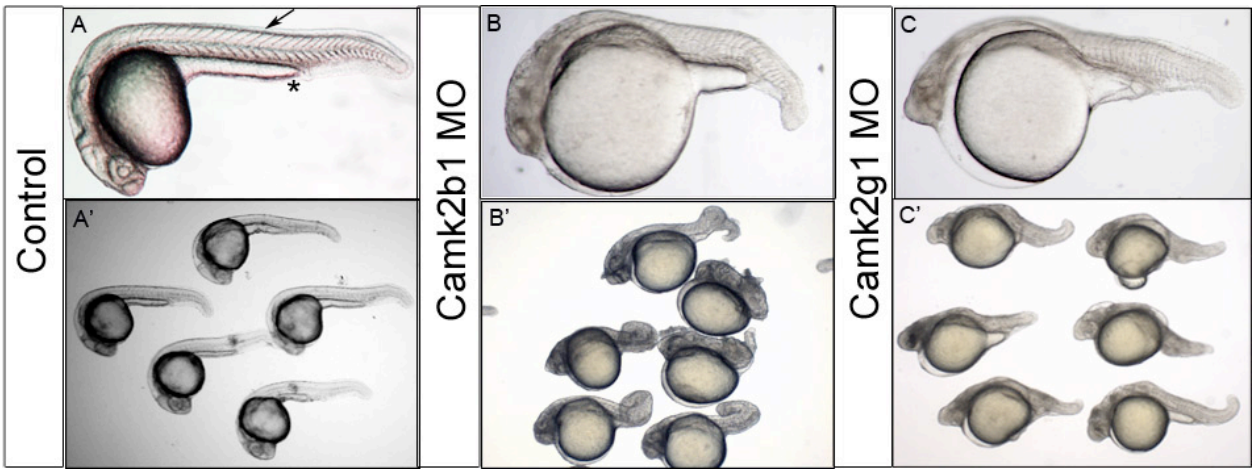


Figure 2-1. Zebrafish *camk2b1* and *camk2g1* morphant phenotypes. (A-C) Lateral view of 24hpf zebrafish embryos using differential interference contrast (DIC) microscopy. (A,B&C) Individual and (A',B'&C') group embryos are shown for each condition. Control embryos show even spaced, chevron shaped somites (arrow) with full yolk extension (asterisk). *camk2b1* morphants exhibit compression of the somites, undulation of the notochord, curvature of the tail and lack yolk extension. *camk2g1* morphants lack yolk and tail extension and exhibit compression of the somites.

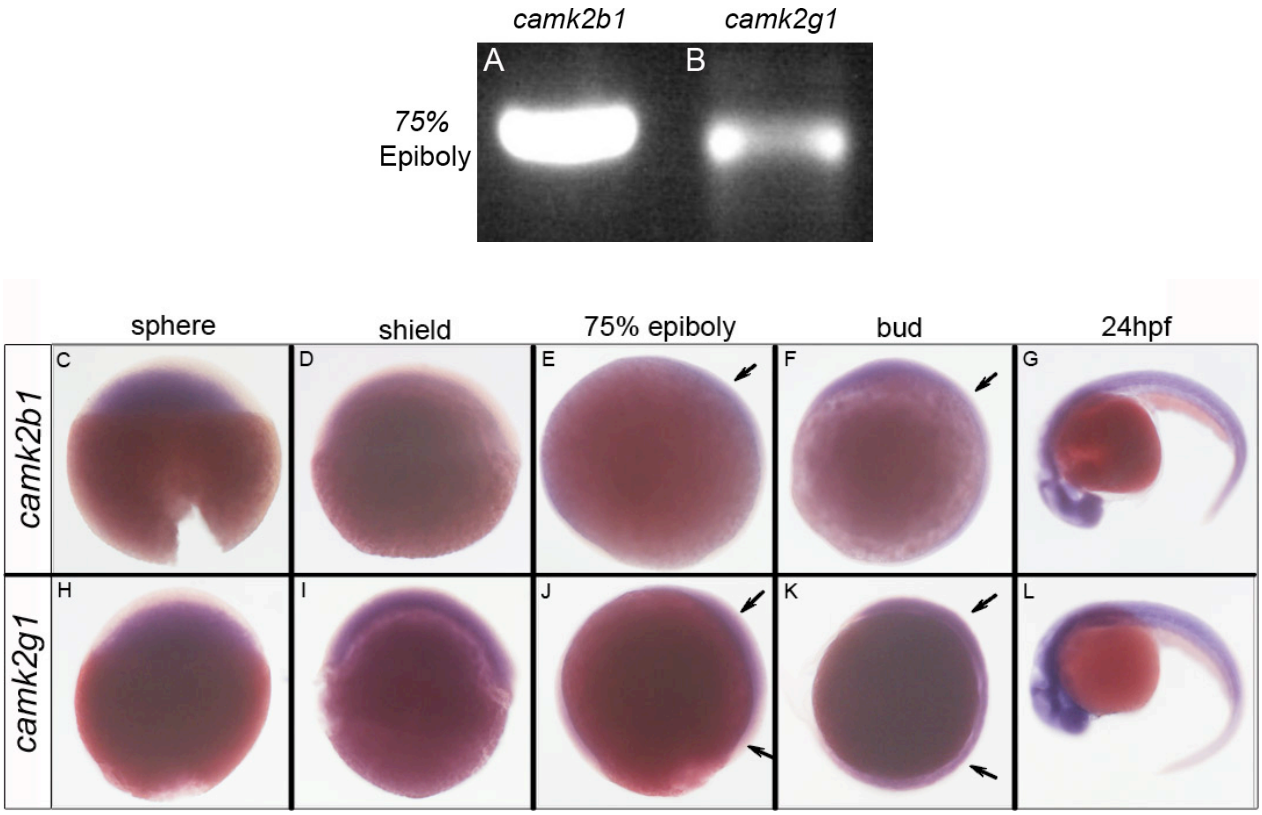


Figure 2-2. *camk2b1* and *camk2g1* expression profile during early zebrafish development. (A-B) RT-PCR of 75% epiboly wild-type zebrafish embryos using *camk2b1* and *cam2g1* specific primers. (C-L) Whole-mount *in situ* hybridization of *camk2b1* and *camk2g1* at the indicated stages. Lateral embryo positioning with anterior (top) and posterior (bottom) (sphere-bud stage), dorsal on the right (shield-75% stage). Arrows indicate anterior and posterior expression (top and bottom, respectively).

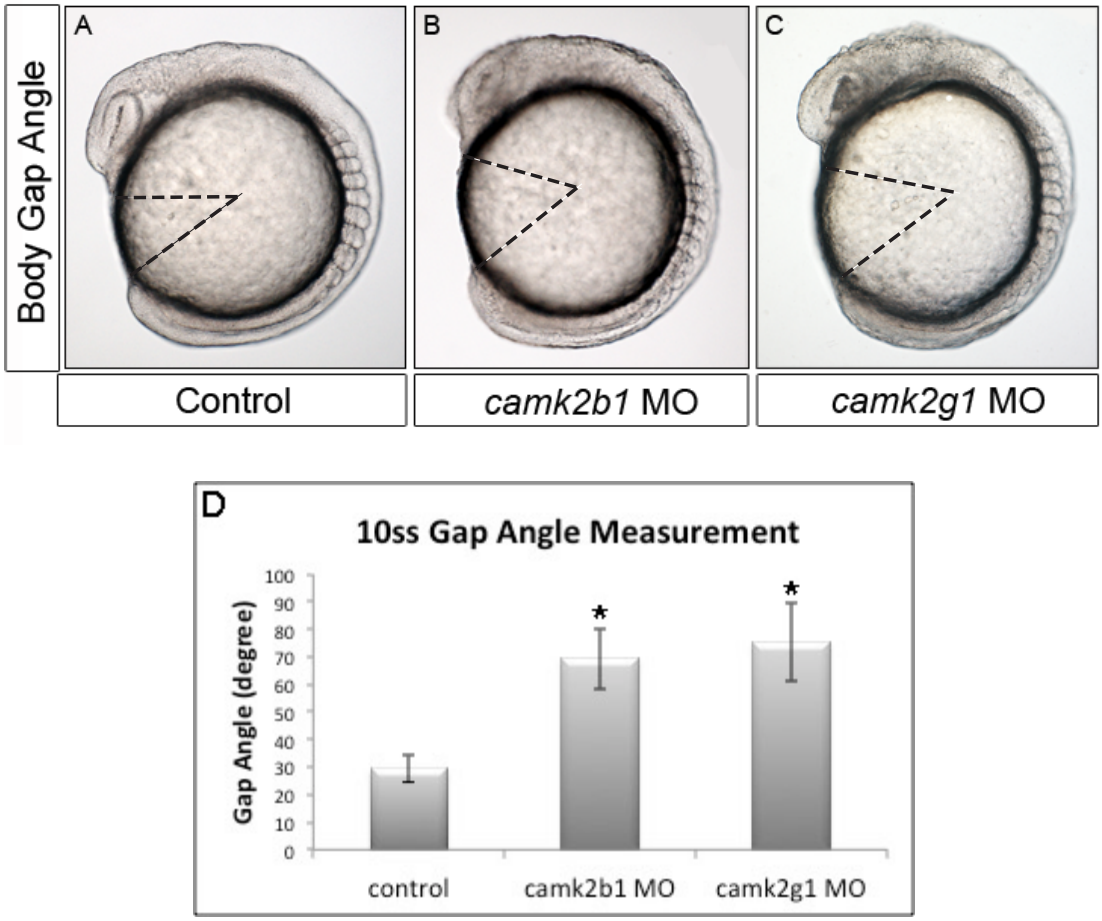


Figure 2-3. CaMK-II morphants exhibit increased 10ss gap angle measurements. (A-C) Lateral view images of representative morphant embryos at 10-11 somite stage (10ss). Body gap angle measured from the head-yolk-tail (dashed lines). (D) Quantification of the average body gap angle of embryos at 10-11 somite stage. Body gap angles were averaged from 34-80 embryos per condition, *P<0.001 compared to control.

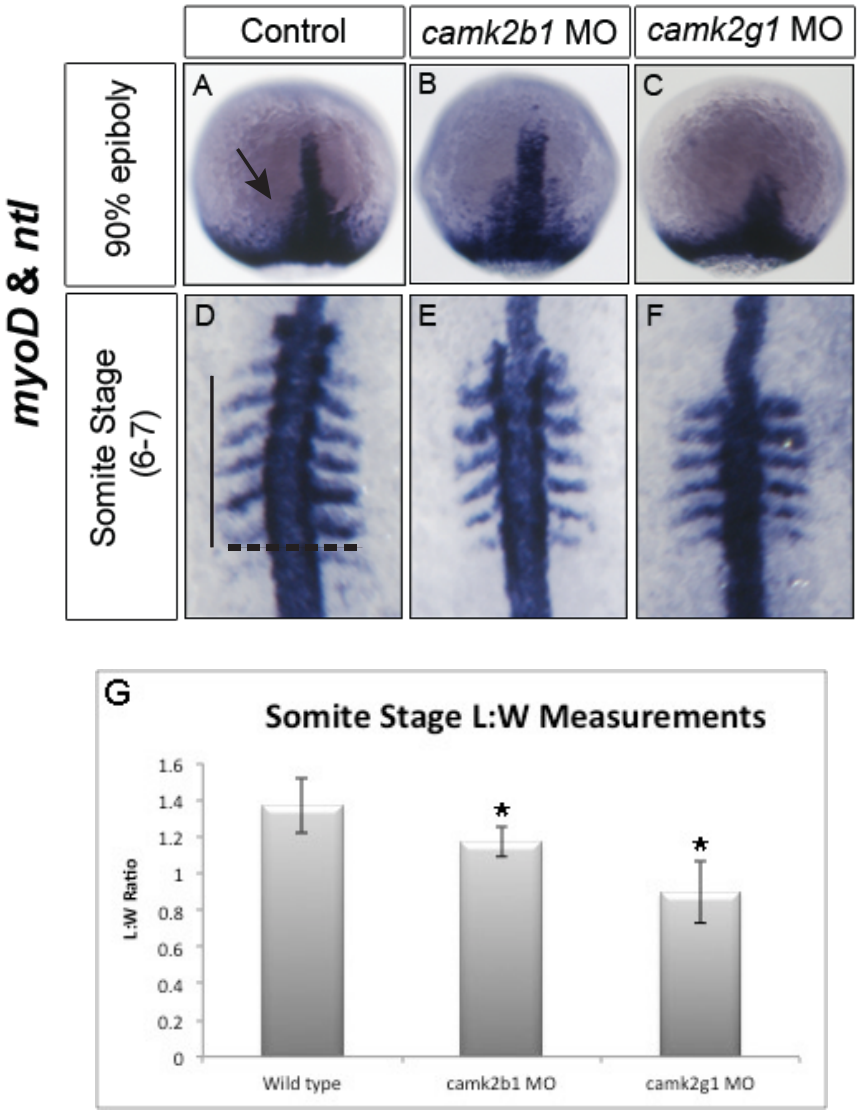


Figure 2-4. *camk2b1* and *camk2g1* morphants display C&E defects of the hypoblast. (A-C) Dorsal view *in situ* hybridized zebrafish embryos with mRNA probes *myoD* & *ntl* at 90% epiboly and (D-F) 6-7 somite stage. (G) Quantification of average length:width (L:W) ratios. Length was measured from the anterior region of somite one to the posterior region of somite 6 (denoted by a solid black line) and from the width of somite 6 (denoted by black dashed lines). L:W ratios were averaged from24-29 embryos per condition, **P*<0.001.

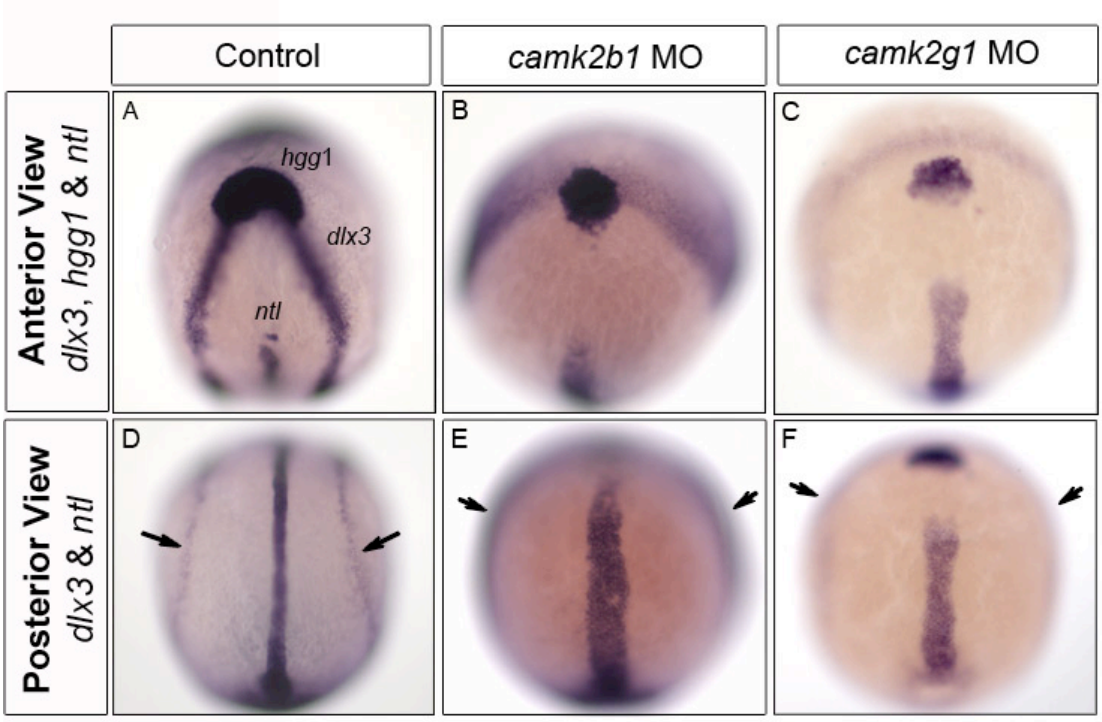


Figure 2-5. *camk2b1* and *camk2g1* morphants display neuroectoderm C&E defects. (A-F) In situ hybridization with combined *hgg1, dlx3* and *ntl* probes. (A-C) Anterior view of dorsal side (D-F) Posterior view of dorsal side. Arrows indicate the width of *dlx3* posterior expression.

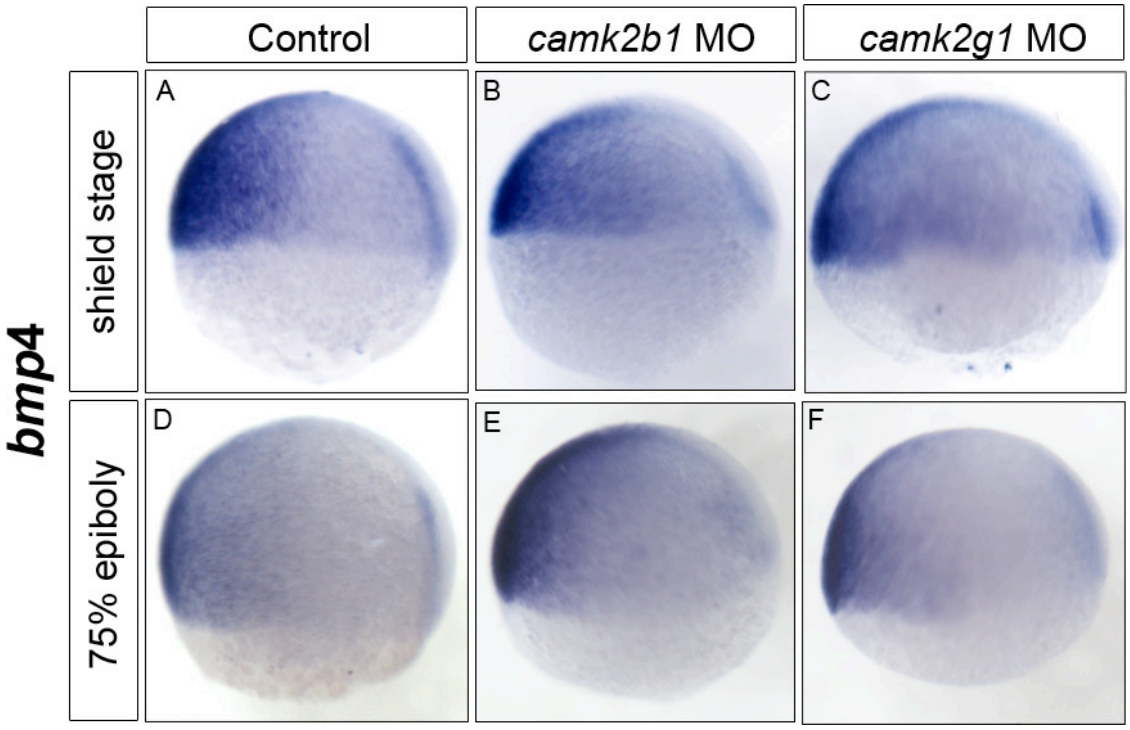


Figure 2-6. Cell fate specification is unaltered in CaMK-II morphants. (A-F) In situ hybridization with probe *bmp4.* (A-C) Lateral view, dorsal to the right shield stage. (D-F) Lateral view, dorsal to the right 75% epiboly.

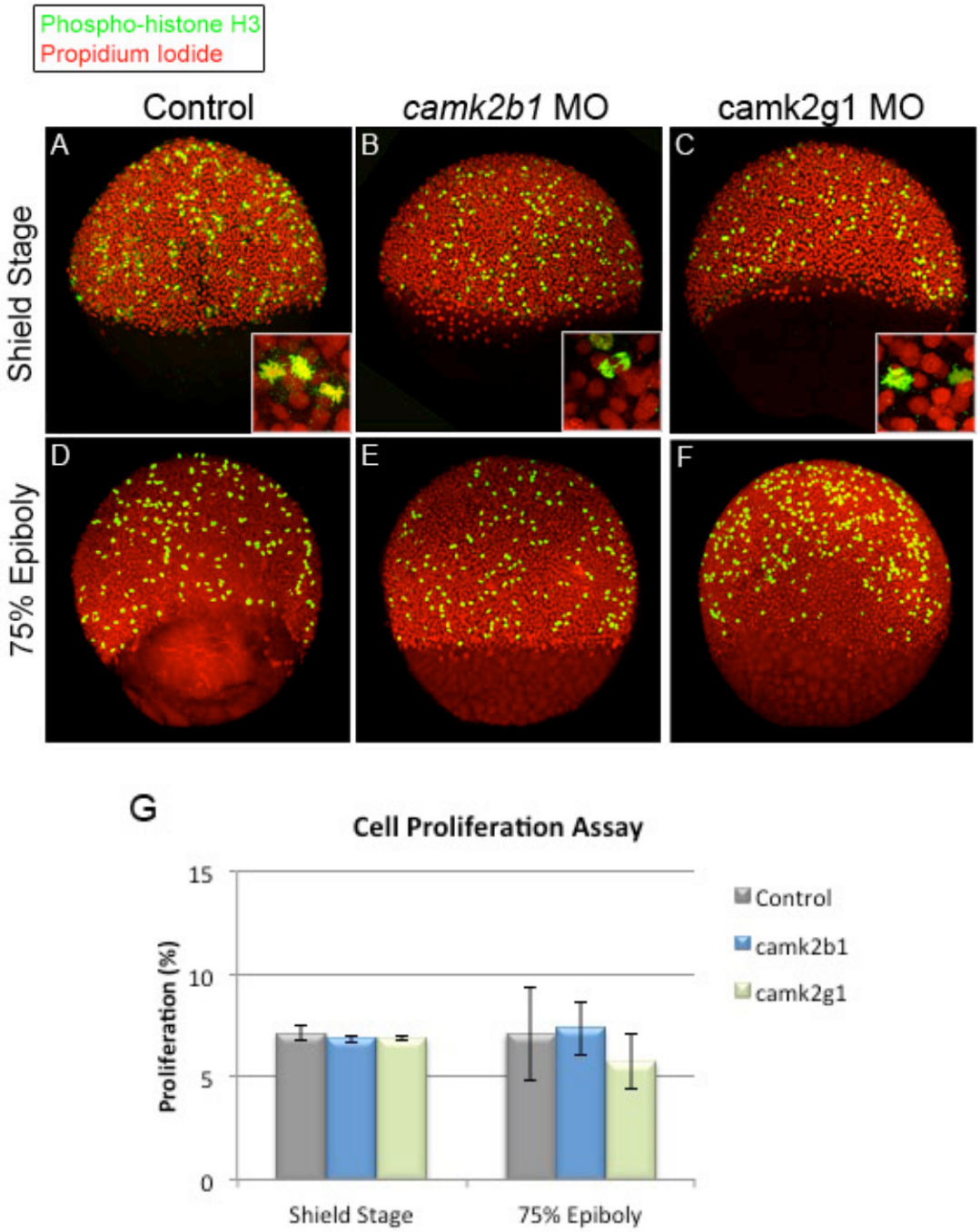


Figure 2-7. Cell proliferation is unaltered in CaMK-II morphants. (A-F) Confocal images of anti-phospho-histone H3 antibody (green) and propidium iodide (red) stained embryos. (A-C) Lateral view, shield stage. Inset show magnified view of actively dividing cells. (D-F) Lateral view, 75% epiboly. (G) Percentage of proliferating cells quantified by counting cells within a 161.5x161.5μM boxed region of the embryo. Averaged from 3 embryos per condition, $P>0.1$ showing no statistical difference.

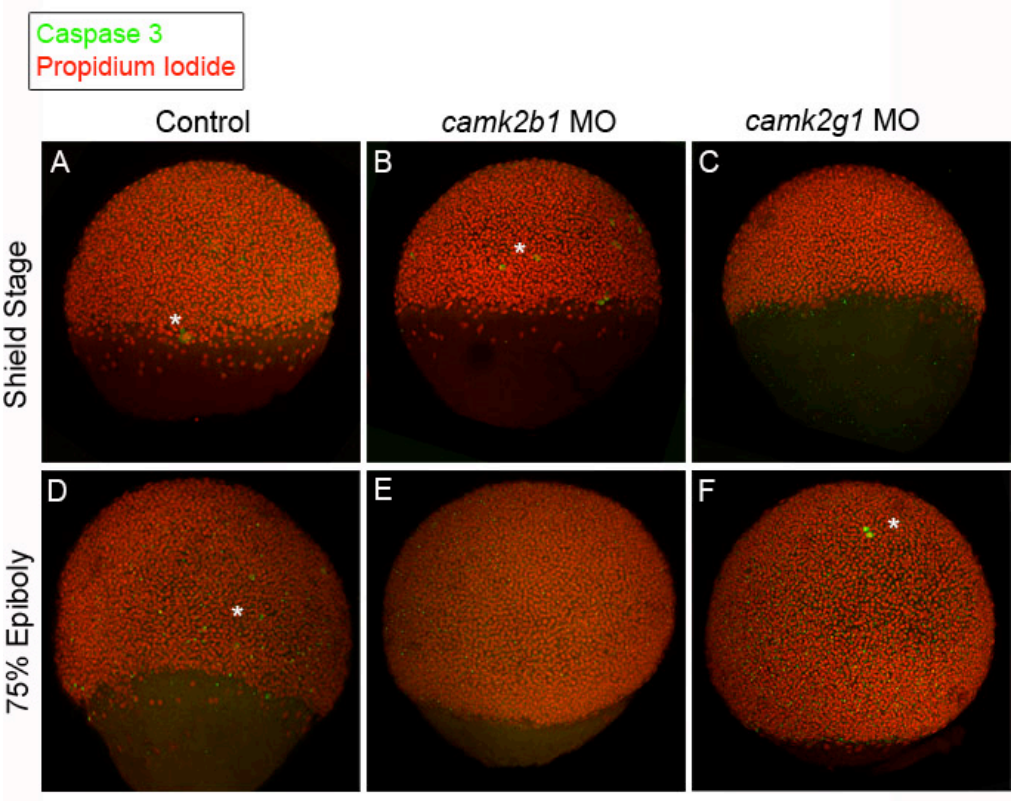


Figure 2-8. Apoptotic cell number is unaltered in CaMK-II morphants. (A-F) Confocal images of anti-Caspase 3 antibody (green) and propidium iodide (red) stained embryos. (A-C) Lateral view, shield stage. (D-F) Lateral view, 75% epiboly. Asterisks indicate individual or clusters of anti-Caspase 3 positive cells.

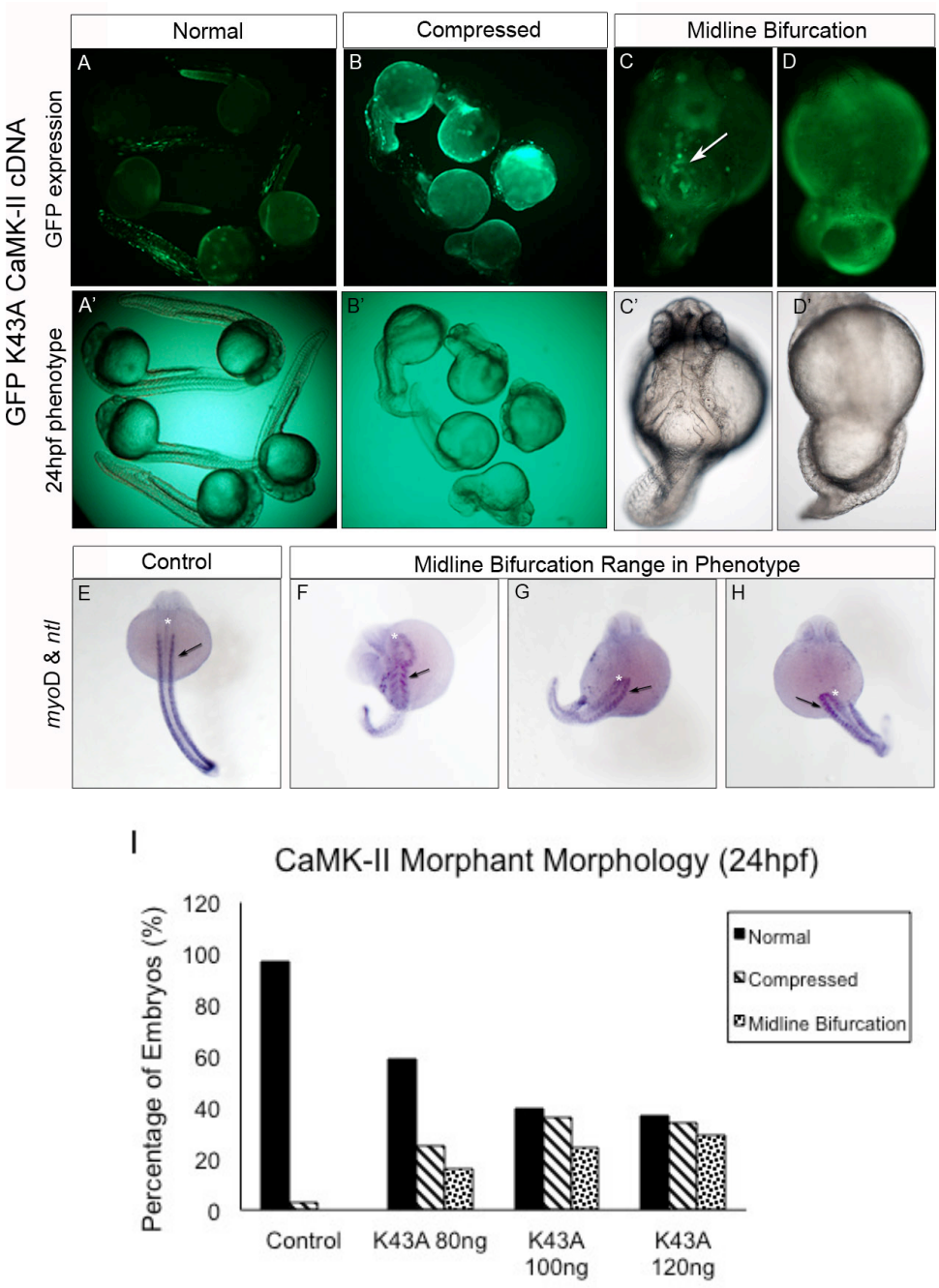


Figure 2-9. Overexpression of dominant negative CaMK-II displays a range of suppressed cell movement phenotypes. (A-H) GFP dominant negative CaMK-II (dnCaMK-II) expressing zebrafish embryos 24hpf. (A-D) GFP expression. White arrow indicates GFP dnCaMK-II positive clustered cells. (E-H) In situ hybridization with digoxigenenin-labeled RNA probes *ntl* & *myoD* at 24hpf. Black arrows denote *myoD* expression and asterisk indicate *ntl* expression.

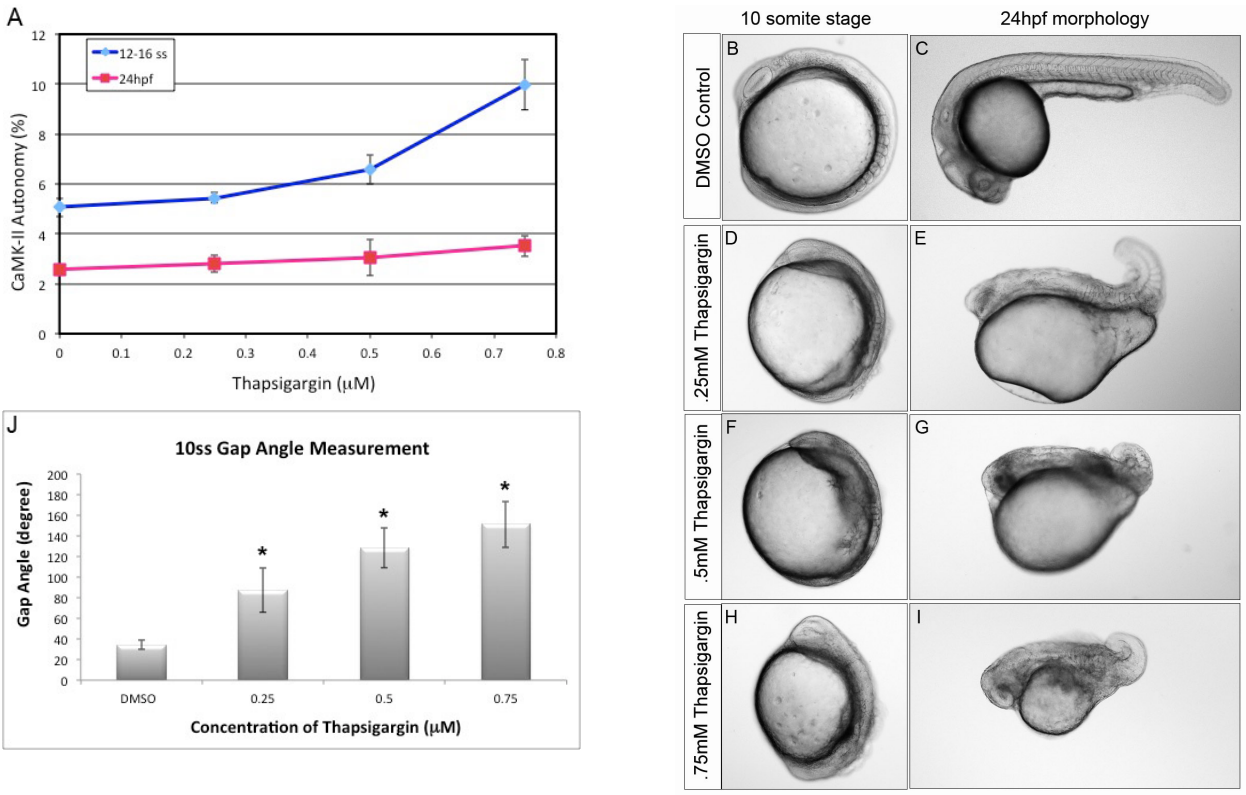


Figure 2-10. Ca^{2+} increase during gastrulation results in CaMK-II activation and cell migration defects. Zebrafish embryos were treated with thapsigargin from 5 to 7hpf. (A) CaMK-II-specific activity (Ca^{2+} independent activity) was measured in whole embryo lysates using the autocamtide-2 peptide–based assay. CaMK-II activity of 12-16 somite stage and 24hpf embryos was measured. (B-I) 10ss and 24hpf phenotypes were assessed of DMSO treated control embryos and 0.25, 0.5 and 0.75μM thapsigargin treated embryos. (J) Body gap angles of control (DMSO) and treated embryos were measured at the10±1 somite stage. (J) Quantification of average gap angle measurements averaged from 17-54 embryos, **P*<0.001.

Chapter 3: Identifying the Mechanism of CaMK-II's Influence on C&E Cell Migration

Introduction

Cell migration during embryonic development is a multifaceted process primarily responsible for the morphogenesis of an embryo. Failure of cells to migrate or migration of cells to inappropriate locations during morphogenesis can result in abnormalities or have life threating consequences (Horwitz and D. Webb, 2003). Key cellular components required to facilitate cell migration include polarity, maintenance of adhesions and the ability to contract and actively move (Horwitz and D. Webb, 2003). Polarity is often reinforced when cells are provided a directional cue such as a chemotactic (induced by chemoattractants or morphogens) or mechanotactic (induced by a breakdown in cell-cell contacts, as in wound healing) (Horwitz and D. Webb, 2003; Parent and Devreotes, 1999). During zebrafish gastrulation, cells to form the embryonic axis are directed to migrate based on a gradient of bone morphogenetic protein (*bmp*); with high to low expression of *bmp* specifying the ventral to dorsal region of the embryo, respectively (Myers et al., 2002a). This gradient of bmp expression is also responsible for facilitating the degree of C&E during gastrulation (Hardt et al., 2007; Myers et al., 2002a).

Changes in cell shape are of central importance for tissue morphogenesis during development. Cell shape is determined by the intracellular components of the cell and the external cytoskeleton (i.e. actin). Control of cell shape, therefore, relies on both the tight regulation of intracellular mechanics and the cells physical interaction with its environment. During vertebrate gastrulation, a driving force of C&E movements requires cells to mediolaterally intercalate. Mediolateral intercalation requires distinct changes in cell shape where axial mesodermal cells must elongate along the mediolateral axis and use

oriented bipolar or monopolar protrusions to drive intercalation between their immediate neighbors (Wallingford et al., 2002). Researchers recently identified myosin II as a required cytoskeleton component necessary for the formation of a cortical actin network in mesoderm cells undergoing mediolateral cell intercalation (Rolo et al., 2009; Skoglund et al., 2008). Interestingly, this cortical actin network consists of foci connected by actin cables, polarized along the mediolateral axis, and undergoes pulsed contractions oriented parallel to the embryonic axis (Paluch and C.-P. Heisenberg, 2009). When myosin II activity is impaired, the cortical actin network is disrupted, cells fail to exhibit normal protrusive activity, and C&E movements are reduced (Paluch and C.-P. Heisenberg, 2009; Skoglund et al., 2008). In addition, interfering with myosin activity also affects cell properties other than contractility, such as cell adhesion (Paluch and C.-P. Heisenberg, 2009; Skoglund et al., 2008). Based on this research, the mediolateral cell intercalations of C&E require clear changes in cell shape; and these changes have been shown to be achieved through regulated cell adhesion, contraction and cytoskeleton dynamics.

Regulation of cytoskeleton dynamics is not only required for the mediolateral intercalations during gastrulation, but also for the epiboly, internalization and convergence & extension cell movements that are also occurring at this stage. Specifically, an actin cytoskeleton network is present in each tissue layer within the gastrula and has been shown to exert varying degrees of regulation on the cell movements. The internal cell layers of the gastrula; ectoderm, mesoderm and endoderm, all contain an actin cytoskeleton which surrounds each cell and is required for their epiboly, internalization and C&E cell movements (Cheng et al., 2004; L L Solnica-Krezel and Driever, 1994; Zalik et al., 1999). An actin cytoskeleton also surrounds each cell of the EVL, with a dense ring of

actin forming at the onset of gastrulation along the EVL margin. In these cells, the actin cytoskeleton has been shown to assist in both facilitating the EVL sheet-like migration as well as providing mechanical tension for cells to be stretched around the yolk cell (Köppen et al., 2006).

This study aims to uncover how CaMK-II may be altering C&E cell movements during zebrafish gastrulation. Previous research has found that cell migration defects associated with inhibition or overexpression of CaMK-II results in distinct changes in cell shape (Easley et al., 2008). Using an *in vitro* wound healing model, researchers found that during normal conditions cells at the leading edge of a wound exhibit a defined and protrusive leading edge with orientation directed towards the open wound area. These directionally oriented cells are elongated along the anterior-posterior (leading and trailing) edges of the cell, and narrowed along the opposite axis. In CaMK-II-deficient or inhibited cells, the leading edge was characteristically irregular, with multiple protrusions and no directed orientation (Easley et al., 2008; Mercure et al., 2008). These changes in protrusions lead to an overall change in cell shape, going from an extended and elongated state under normal conditions to a more rounded and less extended state during CaMK-II inhibition. In this study, CaMK-II's affects on cell migration were shown to be through alterations in the turnover of focal adhesions; the structural link between the actin cytoskeleton and the extracellular matrix of a cell (Easley et al., 2008).

In addition to how CaMK-II is altering C&E cell movements, this work aims to uncover the signaling pathway(s) CaMK-II is working on/through to regulate C&E movements. Of particular interest is the non-canonical Wnt pathway. Zebrafish mutants of the ncWnt signaling pathway include; trilobite (tri) coding for a zebrafish homologue of

Strabismus/Van Gogh like 2 (Vangle2), knypek (kny) encoding a membrane glypican 4, and silberblick (slb) and pipetail (ppt) coding for the secreted ligands Wnt11 and Wnt5/5b, respectively, which all experiencing defective C&E cell movement (C. Heisenberg et al., 2000; Jessen et al., 2002; Rauch et al., 1997; Topczewski et al., 2001). Interestingly, the requirement of ncWnt signaling during gastrulation appears to be important specifically for C&E gastrulation movements; with each of the mutants undergoing normal epiboly, internalization and cell fate specification, while expressing defects of shortened anterior-posterior body axis and widened dorsal structures (somites and notochord) (Roszko et al., 2009). Activation of ncWnt signaling leads to an increase in intracellular Ca^{2+}; with ncWnt mutants displaying a reduction of this Ca^{2+} frequency and/or C&E defects during zebrafish development (Hammerschmidt et al., 1996; C. Heisenberg et al., 2000; Kilian et al., 2003; Slusarski et al., 1997; Westfall et al., 2003). Wnt/Ca^{2+} signaling has previously been shown to regulate tissue separation during gastrulation and may be necessary for cell adhesiveness; an important element of collective cell migration (Lilianna Solnica-Krezel, 2006; Winklbauer et al., 2001).

The core migration machinery of a cell facilitates cell migration. The Rho GTPase protein family has been identified as a crucial component of a cells' core migration machinery; required for the assembly and disassembly of functional cell-cell contacts, such as cadherin and tight junctions, during motility (Bishop and Hall, 2000; Hall, 2005; Schmitz et al., 2000). Rho GTPase's regulate cell migration by acting as molecular switches, cycling between an inactive GDP-bound and active GTP-bound state, with GTPase-activating proteins (GAPs) promoting the inactive state of GTPase (GDP-bound) and Guanine nucleotide exchange factors (GEF's) promoting the activation (GTP-bound) (Schmitz et al.,

2000). Within the Rho GTPase family there are three key members including; Rac, Rho and Cdc42 each of which have been implicated in the establishment of cell-cell contacts and cell-matrix interactions crucial for cell migration (Schlessinger et al., 2009). Interestingly, the Rho GTPase protein family has emerged as a key mediator of the ncWnt pathway, regulating polarized cell shape changes and migrations (Schlessinger et al., 2009). Specifically, Rho GTPases have been implicated as downstream targets of ncWnt-induced signaling to spatially and temporally promote vertebrate morphological development (Schlessinger et al., 2009). During zebrafish development, C&E cell movements have been shown to require Rho GTPase signaling and the C&E defects of ncWnt mutants can be rescued by components of the Rho GTPase protein family (Moeller et al., 2006; Schlessinger et al., 2009; Zhu et al., 2006).

In continuing to understand how CaMK-II influences the C&E cell movements of zebrafish gastrulation, this study assesses the effects a knockdown in CaMK-II has on cell shape to facilitate the C&E defects previously characterized in *camk2b1* and *camk2g1* morphants. Our results find that inhibition of CaMK-II causes significant cell shape alterations within multiple layers of the gastrula, and suggests CaMK-II may be regulating cytoskeleton dynamics to facilitate cell migration during zebrafish gastrulation. In addition to addressing how CaMK-II influences C&E, the signaling pathway CaMK-II is acting on during gastrulation was also identified; linking CaMK-II with ncWnt Wnt11. Finally, CaMK-II's involvement with Rho GTPase signaling was also assessed.

Methods

Zebrafish strains and handling

Animal care and husbandry was performed according to methods previously described (Kimmel et al., 1995). Wild-type (AB and WIK) and transgenic GFP-CAAX embryos were raised at 28.5°C and fed twice daily dry fish food, pellets or Tetra flakes, in combination with live brine shrimp. Zebrafish were reared on a 14:10h light:dark cycle. Water quality was checked during feedings to monitor temperature (25-31°C), conductivity (300-1500mS), and pH (6-8). Ammonia (0ppm(mg/L)), nitrite (0ppm (mg/L)), and nitrate (below 40ppm (mg/L)) levels of the water were measured on a bimonthly basis. The numbers listed represent the desired ranges for each condition. Wild-type zebrafish were bred by natural pairings in breeding tanks and the eggs laid were collected, transferred to embryo water and raised (Kimmel et al., 1995). Microinjections were performed at the one cell stage. All experimental embryos were sacrificed by 48hpf via prolonged immersion in tricane methane sulfonate (MS222, Sigma) 200mg/L, as outlined in the NIH guidelines for use of zebrafish.

Microinjections

Morpholino anti-sense oligonucleotides (MO's) were diluted in Danieau buffer and injected at the 1 to 4-cell stage (Westerfield, 1993). *camk2b1* and *camk2g1* MO's were designed by Gene Tools (Philomath OR) and injected at up to 8ng per embryo. The *camk2b1* MO compliments mRNA sequence from -19 to +6 relative to the start codon (GGCCATGTCTTCCCGTCTCGGACTC). The *camk2g1* MO compliments mRNA sequence from -13 to +12 relative to the start codon (AATTGTAGCCATGGTTGTGTGTGCGT). The *wnt11* MO compliments mRNA sequence from -3 to +22 relative to the start codon (GAAAGTTCCTGTATTCTGTCATGTC). Morpholino stocks (1mM) were stored at -80°C.

Prior to injection, MO aliquots were heated to 65°C for 5 minutes, cooled to room temperature and diluted in Danieau buffer (Westerfield, 1993). Zebrafish wild-type and constitutively active (T287D) CaMK-II was inserted into the pCS2P+ vector, where mRNA was synthesized using the SP6 promoter. All injections were performed at a constant injection volume of ~1nl per embryo, volume analysis was performed for each injection via injection into mineral oil on a micrometer.

Whole mount in situ hybridization

Digoxigenin-labeled anti-sense riboprobes (0.5-1.5kb) were synthesized using T3 or T7 RNA polymerase from cloned cDNAs, hybridized with fixed embryos and then developed using alkaline phosphatase-conjugated anti-digoxigenin antibodies as described (Rothschild et al., 2007). The *ntl* probes was synthesized from cDNA provided by Debbie Garity, PhD. The Thisse lab, at the University of Virginia, performed the combination of *dlx3*, *hgg1* and *ntl* staining.

Immunolocalization

Embryos were fixed in fresh 4% paraformaldehyde for 2-4hrs at room temperature, washed in PBT (1xPBS, tween-20) and incubated in primary antibody. Primary antibodies included rabbit polyclonal antibody against green fluorescent protein (GFP). Goat anti-rabbit Alexa^{488} secondary was used after the primary antibodies.

Phalloidin Staining

Embryos were fixed overnight at 4°C in freshly prepared solution of 4% paraformaldehyde. Prior to staining, embryos were dechorionated and washed several times in PBS for 10 minutes, under gentle shaking conditions. Embryos were incubated with fluro alexa 488 phalloidin (Sigma) for 4-6hrs, washed several times in PBS for 10 minutes and stored/imaged in 50% glycerol in PBS.

Drug Treatment

The JNK inhibitor, SP600125 (sigma) was provided by Amanda Dickinson, PhD. SP600125 was used at concentrations ranging from 1-5uM and a final concentration of 1%DMSO was added directly to the zebrafish culture medium. Embryos were treated from 5-24hpf, washed, and raised inside their chorions until the desired stage of development.

Microscopy

Live embryos were imaged using differential interference contrast (DIC) optics after transient anesthesia with 0.003% Tricaine (MS222, Sigma) and immobilization between coverslips. NIKON 20X, 40X and 100x oil immersion Plan APO objectives were used for DIC and confocal imaging.

Measurements and Statistical Analysis

Length:Width measurements were collected using Nikon Elements software. Cell roundness was measured using imageJ softwarewhich uses the following formula:

$$\text{Roundness} = \frac{4*\text{area}}{\pi*\text{major axis}^2}$$

Statistical analyses of length:width ratios and cell roundness were performed using Microsoft Excel t-test functions. All t-tests were 2-tailed, unpaired, and assuming unequal variance. An asterisk denotes statistically significant differences with *P*-values indicated in figure legends.

Results

Part A: Assessing Cell Morphology

CaMK-II morpholinos block cell migration after early somitogenesis

The C&E defects associated with a knockout or knockdown of the ncWnt genes has been characterized in zebrafish. Researchers found that the phenotype seen in *wnt*5/*ppt* and *wnt*11/*slb* mutants is reproducible using morpholino oligonucleotides (Lele et al., 2001). Using increasing concentrations of morpholino, researchers found that the phenotype of morphants was equivalent or slightly more severe than mutants (Lele et al., 2001). Mutant *camk2b1* or *camk2g1* zebrafish have not been generated and only low CaMK-II morpholino concentrations have been characterized thus far (see Chapter 2). To determine if additional early developmental defects exist during a knockdown in CaMK-II, increasing concentrations of *camk2b1* and *camk2g1* morphants were assessed.

By increasing the concentration of CaMK-II morpholinos (6pg), morphants display a significantly more severe cell migration defect, with development ceasing by late gastrulation (*camk2g1* MO) or early somitogenesis (*camk2b1* MO). The phenotype of *camk2g1* morphants was so severe that the embryos were unable to complete gastrulation, showing incomplete yolk bud closure. The embryos survived for up to 24hpf, yet never developed somites (Fig.3-1B). *camk2b1* morphants displayed a less severe phenotype and

were able to enter into somitogenesis (Fig.3-1A). However, using slightly higher concentrations of *camk2b1* morpholino (8pg) development did not continue past this early somitogenesis stage (data not shown), thus the higher concentration of *camk2b1* morpholino was used for all further experiments.

CaMK-II morpholinos severely disrupt the C&E of axial tissue

Again, using 6 and 8pg of *camk2b1* and *camk2g1* morpholino, the developing axial mesoderm cells was assessed via *in situ* hybridization using the *ntl* probe. These axial cells are responsible for forming the embryonic notochord, which is specified at shield stage and continues to converge and extend throughout gastrulation. Disrupted C&E results in a widening of axial tissue, as previously noted with lower concentrations of *camk2b1* or *camk2g1* morpholino, in addition to ncWnt morphants and mutants (chapter 2) (Hammerschmidt et al., 1996; Myers et al., 2002b). Due to the severe defects shown using high concentrations CaMK-II morphants, axial tissue specification and localization was assessed in bud stage embryos to ensure cell fate specification was properly occurring and to assess the overall phenotype of the developing axial structures.

Control embryos show clear linear expression of *ntl,* spanning from the tail bud region all the way up and around to the anterior region of the developing embryo (Fig. 3-2A). Dorsal view magnified *ntl* images show that the width of *ntl* expression is approximately 2-3 cells wide at bud stage (Fig. 3-2B&B'). CaMK-II morphants show clear disruption of *ntl* expression with *camk2b1* morphants showing lightened expression of *ntl* in the tail bud region of lateral view embryos with expression only reaching approximately half way around the embryo (Fig.3-2C). Dorsal view *camk2b1* morphants show a midline

gap in *ntl* expression, with expression spanning far greater than 2-3 cells in width (Fig.3-2D&D'). *camk2g1* morphants display lateral view expression of *ntl* similar to *camk2b1* morphants, with light expression spanning from the tail bud to approximately halfway around the embryos (Fig. 3-2E). Dorsal view *ntl* expression shows an even greater midline gap in axial expression, in addition to the axial tissue spanning far greater than 2-3 cells (Fig.3-2F&F'). Paraxial cells of these higher concentration CaMK-II morpholino embryos were also assessed using *myoD* probe and *in situ* hybridization. Our results found that while the paraxial tissue was clearly defined in morphants embryos, the expression of *myoD* was greatly widened with dorsal view images of embryos unable to show the full *myoD* expression (data not shown). These results show that axial cell fate specification in high concentration CaMK-II morphants is unaffected; however the midline convergence and anterior-posterior extension of these cells is severely distorted by the end of the gastrulation.

CaMK-II morphants display smaller and rounder ectodermal cells

Misregulation of CaMK-II *in vitro* has previously shown to result in a change in cell morphology; with inhibition of CaMK-II resulting in over stabilization and enhanced cell spreading and constitutively active CaMK-II resulting in a rounded up cell morphology (Easley et al., 2008). In each scenario, cells were unable to migrate. Due to the extreme phenotype experienced using increased concentrations of CaMK-II morpholinos, where development beyond early somitogenesis was inhibited, cell morphology assessments were examined and measurements were collected to determine if morphants experience changes in cell morphology, which may be affecting migration.

The cells and overall morphogenesis of zebrafish embryos was assessed at shield, late gastrulation and early somitogenesis stages, with cell area and cell roundedness measurements taken from shield stage embryos. Control and morphant embryos appear to develop to shield stage fairly normally, with clear defects in *camk2b1* and *camk2g1* morphants not being visible until late gastrulation. By late gastrulation, control embryos show a defined accumulation of cells on the dorsal side where the hypoblast is developing (asterisk) (Fig. 3-3B control), whereas *camk2b1* and *camk2g1* morphants lack this clear dorsal side accumulation of cells (Fig. 3-3E&H).

Dorsal view early somitogenesis images show control embryos with a notochord (n) and chevron shaped somites (arrow) (Fig.3-3C'). In addition, the yolk of control embryos has become more oblong in shape, with the wider region corresponding to the width of the embryo (arrows) (Fig.3-3C). No somites were visualized for either CaMK-II morphant at early somitogenesis (Fig.3-3F'&I'). Dorsal view *camk2b1* morphants display a widened notochord (n) with widened accumulation of paraxial cells; however the cells of the paraxial tissue lack adequate organization to form clearly defined somites (Fig. 3-3F'). The majority of *camk2g1* morphants only developed to the late gastrulation stage however, for the small portion of embryos that appeared to develop beyond late gastrulation (<5%), dorsal view images show no cell organization of the axial or paraxial tissue and only a small posterior cluster of cells, which appears to be forming the tail (Fig. 3-3I &I'). Both CaMK-II morphants display a much more rounded yolk as opposed to the oblong shaped yolk of control embryos (Fig. 3-3F&I).

The overall embryonic phenotype of high concentration CaMK-II morphants appears fairly normal at shield stage, with cells migrating to the appropriate distance

around the yolk cell. However, due to the severe defects displayed by late gastrulation and early somitogenesis morphant embryos, a closer look at individual cell morphology at shield stage embryos was evaluated. Specifically, ectodermal cells of shield stage embryos were visualized using differential interference contrast (DIC) microscopy; cell area and roundedness was measured. The area of shield stage CaMK-II morphant cells was significantly smaller for both *camk2b1* and *camk2g1* ($P<0.001$), with cells characterized by being more rounded in overall morphology (Fig. 3-3J&K).

To conclude, these results show that high concentration CaMK-II morpholino injections result in a loss of dorsal-side cell accumulation by late gastrulation, with severely disrupted or completely lost axial and paraxial structures by early somitogenesis. Cell morphological analysis shows that shield stage morphant embryos exhibit a significant decrease in cell area with a more rounded cell shape.

A knockdown in CaMK-II alters elongation of the enveloping layer cells at late gastrulation

Proper cell morphology is critical during cell migration. Directed cell migration is facilitated by a change in cell shape, with cells orienting themselves toward the area of migration. During zebrafish gastrulation, multiple modes of cell migration are simultaneously observed thus, assessment of individual cell layers can provide great insight into how cell movements are occurring and if defects are present.

As previously noted, a loss in CaMK-II results in defective C&E movements. While it is clear that the EVL does not participate in C&E cell movements during gastrulation, the EVL does experience a change in cell shape at the same time C&E movements are taking place (Rohde and C.-P. Heisenberg, 2007; Warga and Kimmel, 1990). Specifically, by late

gastrulation, EVL cells become stretched and elongated along their anterior-posterior axis to help in facilitating the closure of the yolk plug (Rohde and C.-P. Heisenberg, 2007). It has been previously suggested that during gastrulation the EVL and underlying cell layers may interact with one another, in addition to the YSL, in driving the cell movements of the EVL and internal blastomeres (Rohde and C.-P. Heisenberg, 2007). Thus, to determine if a defect in C&E of the internal cell layers results in cell shape changes of the external cell layer, we investigated cell morphology of the EVL in CaMK-II morphants.

By late gastrulation the EVL has thinned and cells are elongated and oriented toward the vegetal pole (Köppen et al., 2006). In these studiens, phalloidin staining of the actin filament was used to assess cell shape and calculate L:W ratios of the EVL cells at late gastrulation. Representative cell tracings show that the EVL cells of CaMK-II morphants appear to have an overall more rounded cell morphology at late gastrulation (Fig. 3-4A''B''C''). The L:W ratio of individual cells was taken along the vertical (length) and horizontal (width) axis of the cell. Our results showed morphant embryos displayed significantly smaller ratios from the controls (Fig.3-4D). In addition to assessing EVL cell shape; we examined the actin ring which forms at the onset of gastrulation, connects to the YSL and has been shown to be required for EVL migration (Köppen et al., 2006; Rohde and C.-P. Heisenberg, 2007). Phalloidin staining detected no significant difference between the control and CaMK-II morphant actin rings, with each localizing to the EVL margin and distributed uniformly around the embryo (asterisk) (Fig.3-4A,B&C). To summarize, these results show that the cell morphology of the EVL is misregulated during a knockdown in CaMK-II and that these changes are not due to a disruption in the cortical actin ring at the EVL margin.

CaMK-II is essential for the elongation of paraxial mesoderm cells during convergent extension

Mediolateral cell intercalation behavior (MIB) occurs when axial and paraxial mesoderm cells elongate along the mediolateral axis and drive intercalation between neighboring cells to facilitate the convergence and extension of mesoderm tissue (Wallingford et al., 2002). Proper C&E requires MIB, thus to determine if MIB is altered in CaMK-II morphants; paraxial mesoderm cells were analyzed for potentially disrupted cell morphology. Transgenic GFP-CAAX zebrafish embryos, which express membrane GFP in all cells, where used to visualize cell shape of axial mesoderm cells at bud stage embryogenesis. Embryos were fixed between bud and 1-somite stage, antibody stained against GFP to clearly mark the GFP-CAAX cells and imaged. Axial tissue is clearly detected in each embryo, with the axial tissue of morphants widened (arrows) (Fig.3-5A&B).

Cell roundness is the expression of cell elongation (Kilian et al., 2003). Paraxial cell roundness was measured using imageJ software. Paraxial cells (within 6-cells of the axis) in dorsal view DIC images of control, *camk2b1* and *camk2g1* morphant embryos were traced and assessed for roundness (representative image shown Fig.3-5C). Cell roundness was scored between 0 and 1, with 1=round. Control embryos presented an average cell roundness of 0.523±0.12, while *camk2b1* morphant*s* exhibited an average roundness of 0.7095±0.12 and *camk2g1* morphants 0.743±0.13. Both morphants display a significant increase in roundness when compared to control cells $P<0.001$. A scatter plot was used to graph these results (Fig.3-5C). The increase in cell roundness experienced in CaMK-II morphants suggests that a knockdown in CaMK-II is affecting cell elongation necessary for MIB when facilitating C&E.

Part B: Signaling Mechanism

Overexpression of CaMK-II partially rescues the C&E defects of Wnt11 Morphants

The Wnt/Ca^{2+} ncWnt signaling pathway has been identified as a necessary regulator of vertebrate development (Kühl et al., 2000b; Matsui et al., 2005; Rohde and C.-P. Heisenberg, 2007). Overexpression of ncWnt signaling components; Wnt5b, Wnt11 or Fz, during vertebrate development induces an increase in the frequency of intracellular Ca^{2+} release (Sheldahl et al., 1999; Slusarski et al., 1997). An increase in intracellular Ca^{2+} during vertebrate gastrulation has been shown to activate Ca^{2+} sensitive enzymes such as PKC and CaMK-II, to regulate cell adhesion and movement during gastrulation (Kühl et al., 2000a; Sheldahl, 2003; Sheldahl et al., 1999). Finally, overexpression of a truncated constitutively active CaMK-II construct has been shown to partially rescue the C&E defects of Wnt5*/ppt* mutant embryos (Westfall, 2003).

To determine if CaMK-II can rescue additional non-canonical Wnt morphants, zebrafish CaMK-II mRNA was overexpressed in Wnt11 morphants. Previously, researchers used a truncated constitutively active form of CaMK-II when rescuing the C&E defects of Wnt5/*ppt* mutants; however this construct lacks the association domain of CaMK-II and may not necessarily recapitulate the true activity of CaMK-II. Using a combination of wild type and constitutely active zebrafish CaMK-II (2:1 ratio, respectively), overexpression of CaMK-II was used to rescue the C&E defects of Wnt11 morphants. 1-3 somite stage (ss) zebrafish embryos were assessed by *in situ* hybridization using the *dlx3*/*hgg1*/*ntl* combination of probes. C&E was classified into three groups: C&E defect, partial C&E defect, no C&E defect. Defective C&E embryos were defined as showing expression of *hgg1*

posterior to *dlx3*, partially defective C&E expressing *hgg1* on/within *dlx3* expression, and no C&E defect expressing *hgg1* completely anterior to *dlx3* expression (Fig.3-6). Our results show that control and overexpression of CaMK-II shows predominantly no C&E defects (Fig. 3-6A&B). Wnt11 morphants resulted in >60% of the embryos experiencing defective C&E (Fig.3-6C&E). The combination of CaMK-II in Wnt11 morphants resulted in >50% of the embryos experiencing partial C&E defects, ~30% presenting no C&E and less then 20% showing defective C&E (Fig.3-6D&E). Taken together these results show that overexpression of CaMK-II in Wnt11 morphants results in a partial C&E rescue of Wnt11 morphants.

CaMK-II does not interact with JNK to facilitate C&E during zebrafish gastrulation

A downstream target of the ncWnt pathway is Dishevelled (Dsh), the activation of which results in a Ca^{2+} flux and the activation of the Rho GTPases Cdc42, Rac and Rho (Schlessinger et al., 2009; Sheldahl, 2003). During zebrafish development, inhibition of Dsh induces C&E defects and rescue from these problems requires co-expression of the downstream effectors, Rac and Rho (Moeller et al., 2006). Rac signaling is important for regulating actin protrusions facilitating cell migration, in addition to activating c-Jun N-terminal kinase (JNK), the signal transducer and activator of transcription 3 (Stat3) and Prickle1 which have each been shown to be required for normal C&E movements during gastrulation (Carreira-Barbosa et al., 2003; Seo et al., 2010; Takeuchi et al., 2003; Yamashita et al., 2002). Rho signaling involves the activation of Rho-associated protein kinase (ROCK), which has been shown to regulate the shape and movement of cells by

acting on the actin cytoskeleton. Expression of Rho has been found to partially rescue both Wnt11 and Wnt5 zebrafish mutants (Zhu et al., 2006).

To determine if CaMK-II in regulating C&E during zebrafish development in conjunction with the Rho GTPase's, Rac and Rho; a synergism experiment was set up using drugs to target the primary downstream effectors of Rac and Rho signaling, JNK and ROCK, respectively. The synergism of either pathway with CaMK-II was tested using CaMK-II morpholino with either JNK inhibitor (SP600125) or ROCK inhibitor (Y-27632).

The JNK inhibitor was found to induce C&E defects at a concentration of 5μM, displaying compressed somites and tail truncation, with no defects at a concentration of 1μM (Fig.3-7A&B). *camk2b1* and *cam2g1* morpholino show minor C&E defects at 2ng and 1ng, respectively (Fig.3-7E&F). In combining each low concentration CaMK-II morpholino with low concentration SP600125, no additional C&E defects were detected (Fig.3-7E&F). While previous research identified the ROCK inhibitor, Y-27632, as capable of inhibiting ROCK and resulting in C&E defects of zebrafish gastrulation, we were unable to repeat these results and thus could not accurately determine synergistic effects of ROCK with CaMK-II morphants.

Discussion

The results presented here show that at high concentrations, *camk2b1* and *camk2g1* morpholinos can completely block the migration necessary for embryonic morphogenesis through gastrulation. In addition, this work identifies the cell morphological changes that occur during strong inhibition of CaMK-II, and suggests that CaMK-II's influence on cell migration may be occurring via disruption of actin cytoskeleton dynamics. Our findings

also link CaMK-II's effects on cell migration with signaling pathways previously shown to regulate C&E during gastrulation; including ncWnt and Rho GTPase signaling. Overall, this project is the first to show a complete loss in development due to inhibited C&E cell movements during gastrulation and identifies CaMK-II as a required protein for cell migration, downstream the non-canonical Wnt signaling pathway and working in parallel with select Rho GTPase effectors.

High concentration *camk2b1* and *camk2g1* morphant phenotypes demonstrate that CaMK-II is required to facilitate cell migration through gastrulation, with high levels of inhibition resulting in a complete block of morphogenesis (Fig.3-1 and data not shown). Previous research depicting a delay or arrest in zebrafish development, found that the arrest was due to alterations in epiboly cell movements with the convergence & extension of cells occurring normally and specification of cell fate unaltered (Liu et al., 2011; Nornes et al., 2009; Yu et al., 2011). In deducing their results, these researchers showed that the arrested development phenotypes was specifically due to a loss in epiboly cell movements based on the presence and correct localization of axial mesoderm tissue, with convergence and extension of the tissue appearing normal (Yu et al., 2011). CaMK-II has already been characterized as a necessary protein for the C&E cell movements of zebrafish gastrulation, thus to determine if the phenotype of high concentration CaMK-II morphants was due to CaMK-II also affecting epiboly cell migration, axial mesoderm was visualized. Assessment of the axial mesoderm marker *ntl* in high concentration *camk2b1* and *camk2g1* morphants showed an even more severe defective C&E phenotype than previously noted at moderate CaMK-II morpholino concentrations. Specifically, the axial mesoderm tissue showed a decrease in extension with incomplete convergence at the midline resulting in a gap in axial

tissue formation (Fig.3-2). Based on these data, it is believed that CaMK-II acts primarily on C&E cell movements in regulating cell migration during gastrulation and all together these finding show for the first time an arrest in zebrafish development due specifically to defects in C&E cell movements.

The lack of cell migration observed in high concentration CaMK-II morphants is occurring in junction with altered cell shape throughout the developing embryo including: the ectoderm, mesoderm and EVL cell layers. Ectoderm cells of the gastrula display a more round and smaller phenotype suggesting a decrease in cell spreading and misregulation of cell orientation (Fig.3-3). Axial mesoderm cells were analyzed at the end of gastrulation, during bud stage, to determine if MIB in morphants was altered. Morphant axial cells show a decrease in L:W ratio, thus improper MIB (Fig.3-5). Finally, the EVL was investigated and our results found that CaMK-II morphants lack the classic elongated phenotype of wild-type embryos, yet display complete coverage around the yolk suggesting that the altered phenotype is due to interactions between the EVL and underlying blastomeres and is not due to a disruption in the mechanics of EVL migration (Fig.3-4).

In addition to changes in cell shape, as internal blastomeres of the zebrafish embryo migrate to their respective locations during gastrulation, forces are exerted onto the underlying yolk altering overall yolk cell morphology. Upon specification of the anterior-posterior axis, cells of the blastoderm wrap around the yolk cell, exerting force on the yolk in a ring-link fashion and resulting in a change in yolk cell shape from rounded during the blastula and early gastrula stages to more oblong by late gastrulation and early somitogenesis stages (D'Amico and Cooper, 2001; L L Solnica-Krezel and Driever, 1994). High concentration CaMK-II morphants display no apparent change in yolk cell

morphology between shield stage and early somitogenesis stage embryos (Fig. 3-3). It is possible to hypothesize that this lack in yolk cell shape change is yet another indicator that CaMK-II morpholinos are inhibiting the internal blastomeres ability to actively migrate during gastrulation, specifically converge and extend.

With CaMK-II clearly defined as a regulator of C&E cell movements and the ncWnt pathway previously identified as the primary signaling cascade necessary for regulating C&E, testing CaMK-II's ability to rescue the defects of non-canonical Wnt11 was a logical step for these studies. While a complete rescue of the C&E defects of Wnt11 was not achieved through the overexpression of CaMK-II mRNA in these morphants, a distinct partial rescue was observed (Fig.3-7). Non-canonical Wnt signaling is a multifaceted signaling cascade with a plethora of downstream effector pathways. Thus, the idea of CaMK-II being the primary target of non-canonical Wnt signaling to facilitate C&E was unlikely and the partial rescued observed is a much more plausible result; especially since previous researcher have shown that Wnt5 is also only partially rescued during over-expression of a truncated constitutively active CaMK-II construct (Westfall, 2003)

In designing the model for how CaMK-II regulates C&E defects, a synergism experiment was performed using inhibitors against JAK and ROCK, which regulate two separate pathways downstream ncWnt activation, each shown to be important in facilitating proper C&E during development. JNK activation occurs downstream Rac, to facilitate cell migration, in addition to Rac directly affecting actin protrusions necessary for cell migration. Rok activation occurs downstream Rho activation and is necessary for actin-myosin contraction responsible for cell migration. The results of these experiments showed that CaMK-II is working in parallel with JNK signaling in facilitating C&E

movements of zebrafish gastrulation (Fig.3-8&9). On the other hand, inconclusive results were obtained from the ROCK inhibitor experiments, thus continued assessment will need to be made in order to determine synergism.

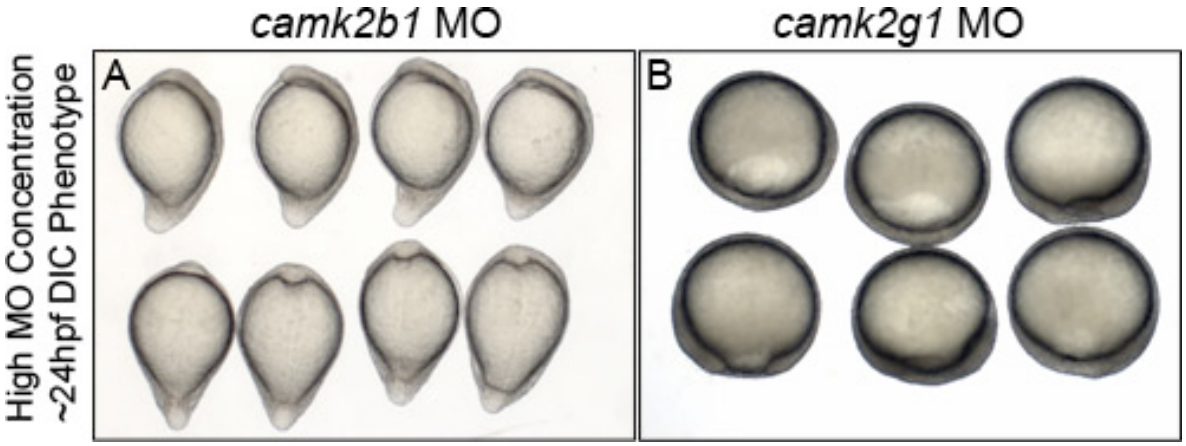


Figure 3-1. CaMK-II morpholinos block cell migration. (A&B) Differential Interference Contrast (DIC) images of increased concentration CaMK-II morpholinos at ~24hpf. (A) 8pg *camk2b1* morpholino. Lateral view (top row), dorsal view (bottom row). (B) 6pg *camk2g1* morpholino. Lateral view.

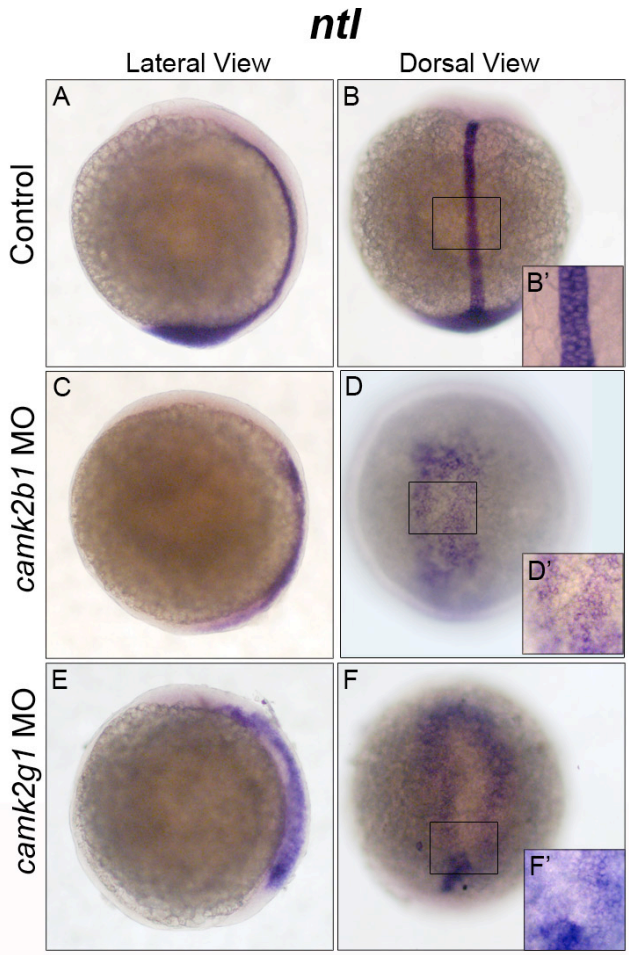
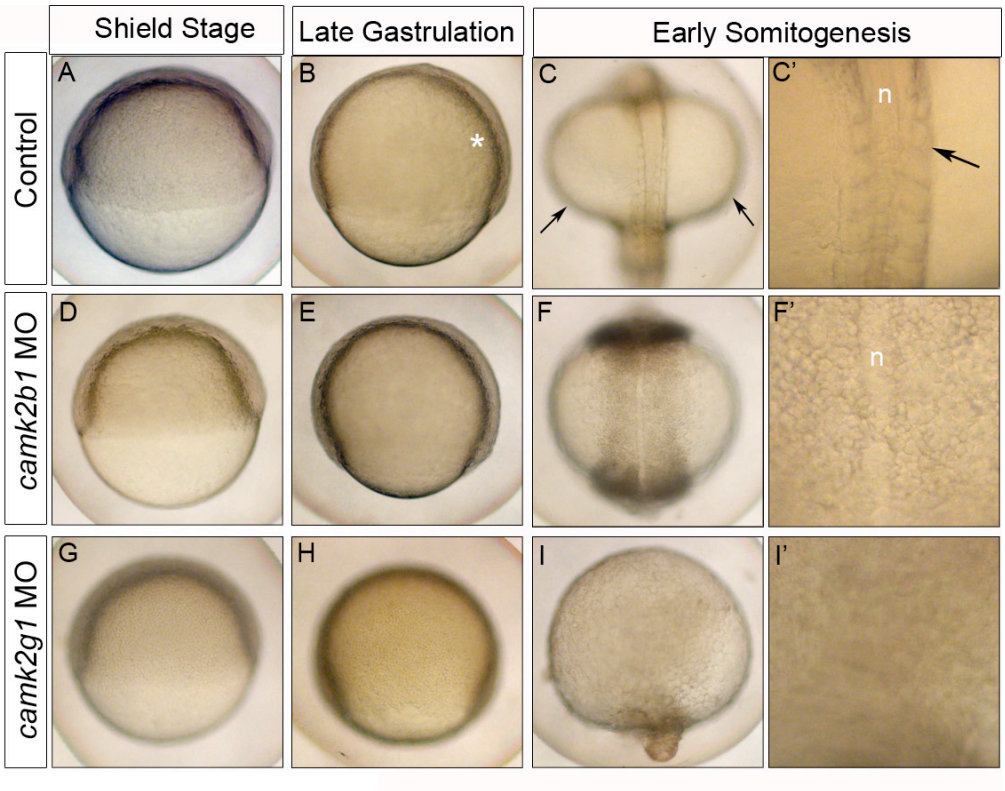
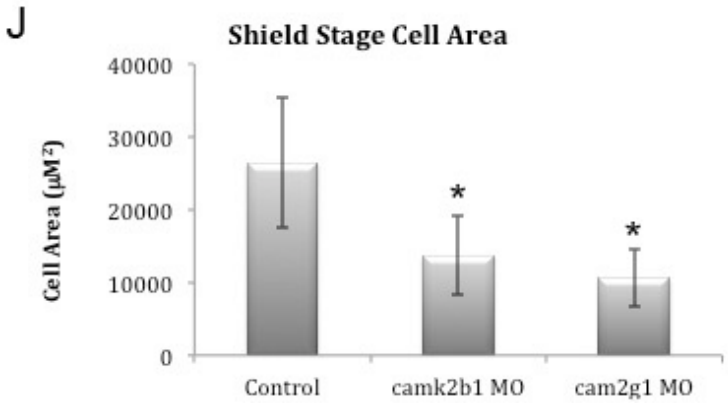


Figure 3-2. CaMK-II morpholinos severely disrupt C&E of axial tissue. (A-F) In situ hybridization of control, *camk2b1* (8pg) and *camk2g1* (6pg) morphants with digoxigenenin-labeled RNA probe *ntl* at bud stage. (A,C,E) Lateral view, dorsal to the right. (B,D,F) Dorsal view, anterior to the top posterior to the bottom. (B',D',F') High magnification dorsal view.


Shield Stage
Late Gastrulation
Early Somitogenesis
Control
camk2b1 MO
camk2g1 MO
A
B
C
C'
D
E
F
F'
G
H
I
I'
*
n
n


J
Shield Stage Cell Area
Cell Area (μM²)
40000
30000
20000
10000
0
*
*
Control
camk2b1 MO
cam2g1 MO

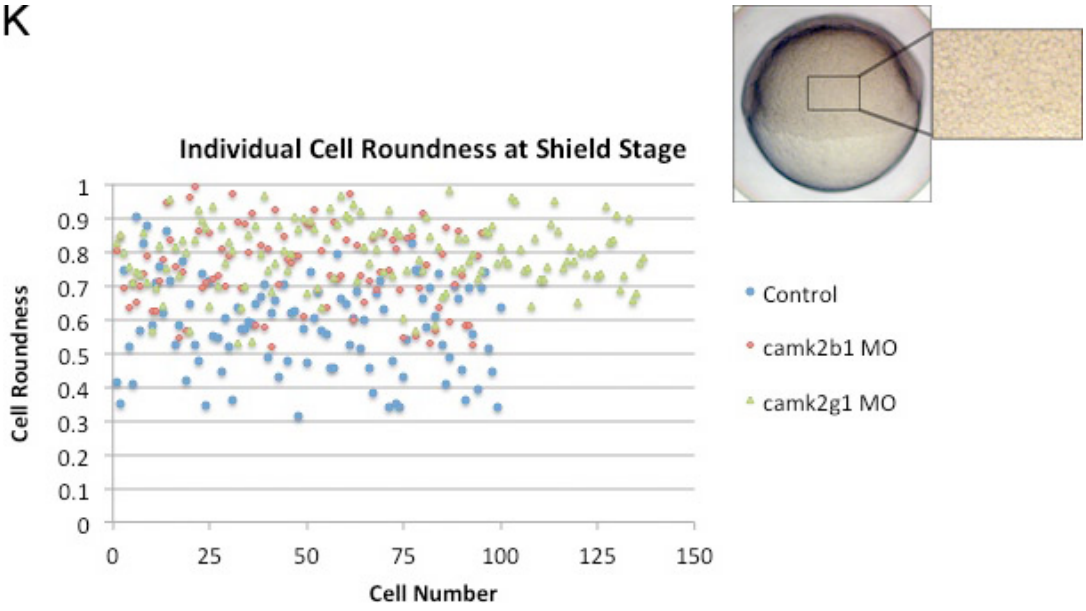
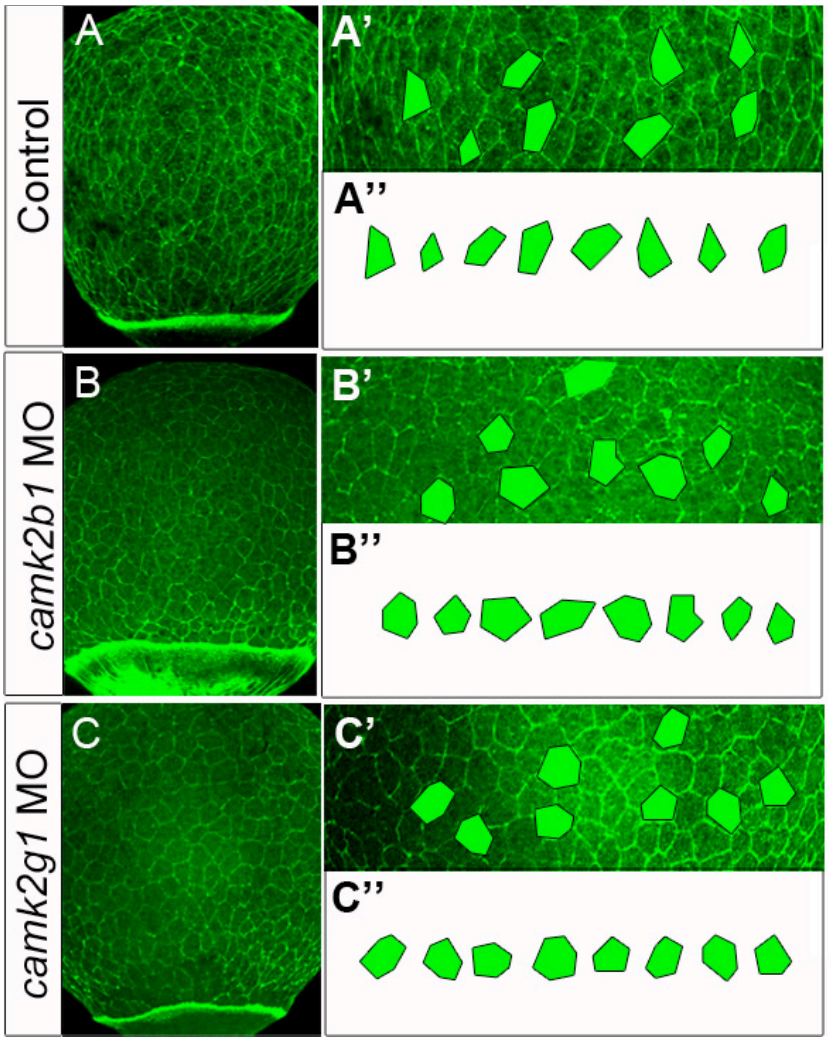


Figure 3-3. CaMK-II morpholinos result in smaller and rounder ectoderm cells. (A-I) Differential Interference Contrast (DIC) images of control and increased concentration CaMK-II morpholinos at shield, late gastrulation and early somitogenesis stages. Control morpholino, 8pg *camk2b1* morpholino and 6pg *camk2g1* morpholino. (A,B,D,E,G,H) Lateral view, dorsal to the right. (C,F,I) Dorsal view, anterior to the top. (C',F',I') High magnification individual cell morphology). White asterisk denotes developing hypoblast. (C) Arrows indicate width of yolk cell (C') Arrow indicated chevron shaped somite. (C',F') n=notochord. (J) Quantification of cell area averaged from 3 embryos per condition, with a total of 85-177 individual cells measured per condition. **P*<0.001. (K) Cell roundness was averaged from 3 embryos per condition, with a total of 95-137 individual cells measured per condition. **P*<0.001.


Control
A
A'
A''
camk2b1 MO
B
B'
B''
camk2g1 MO
C
C'
C''

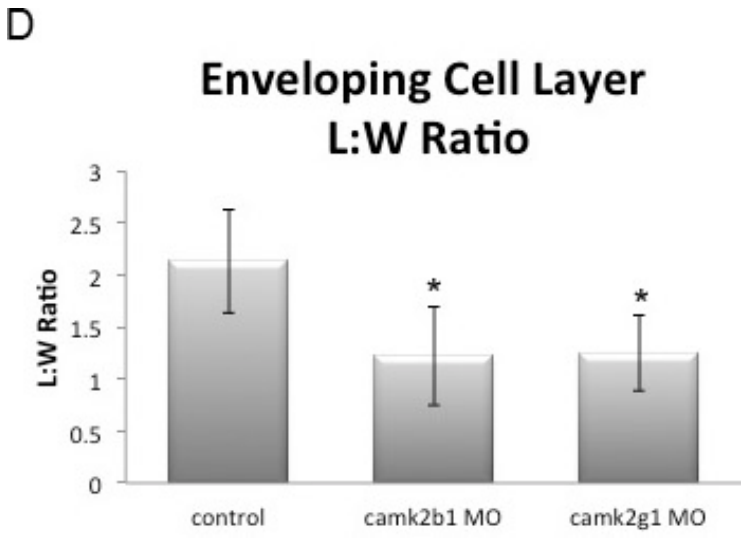
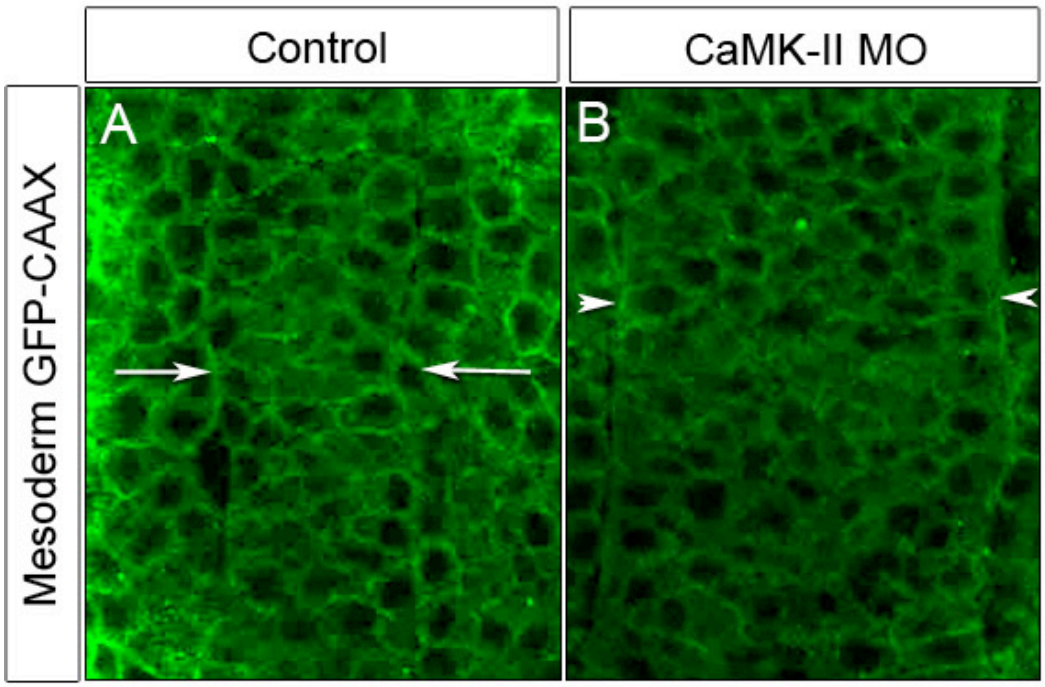
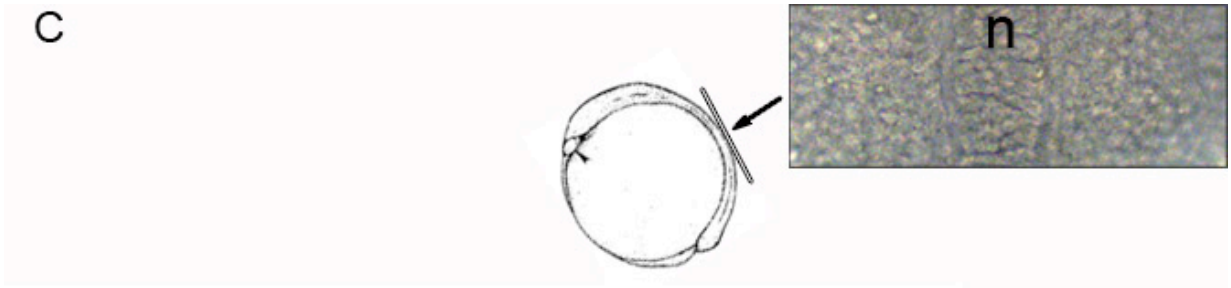

D
Enveloping Cell Layer
L:W Ratio
L:W Ratio
3
2.5
2
1.5
1
0.5
0
*
*
control
camk2b1 MO
camk2g1 MO

Figure 3-4. A strong knockdown of CaMK-II alters elongation of the enveloping layer cells at late gastrulation. (A-C") Alexa fluor 488 phalloidin stained EVL cells at late gastrulation. (A'-C") Magnified view and representation of individual cell tracings of control, *camk2b1* and *camk2g1* morpholinos. (D) Quantification of average L:W ratios calculated from 3 embryos per condition, with a total of 85-218 cells measured per condition. **P*<0.001.


Control
CaMK-II MO
Mesoderm GFP-CAAX
A
B


C
n

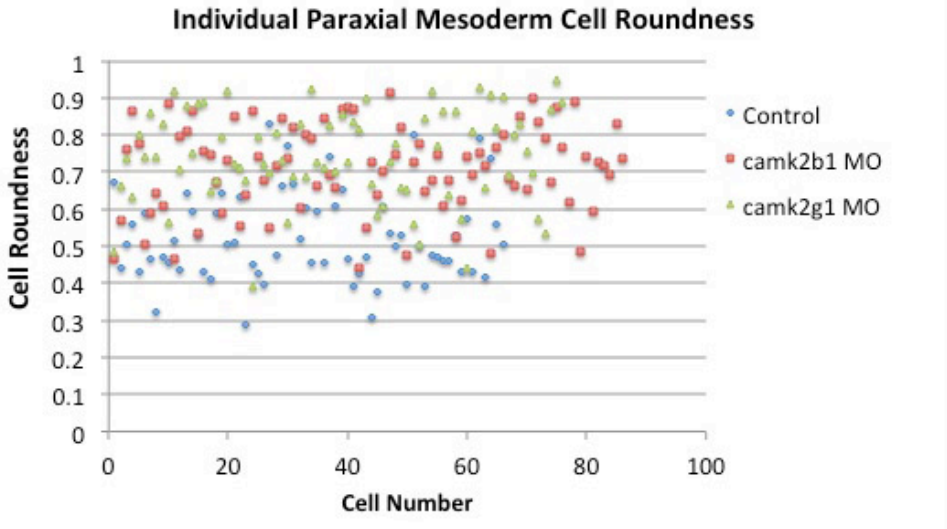
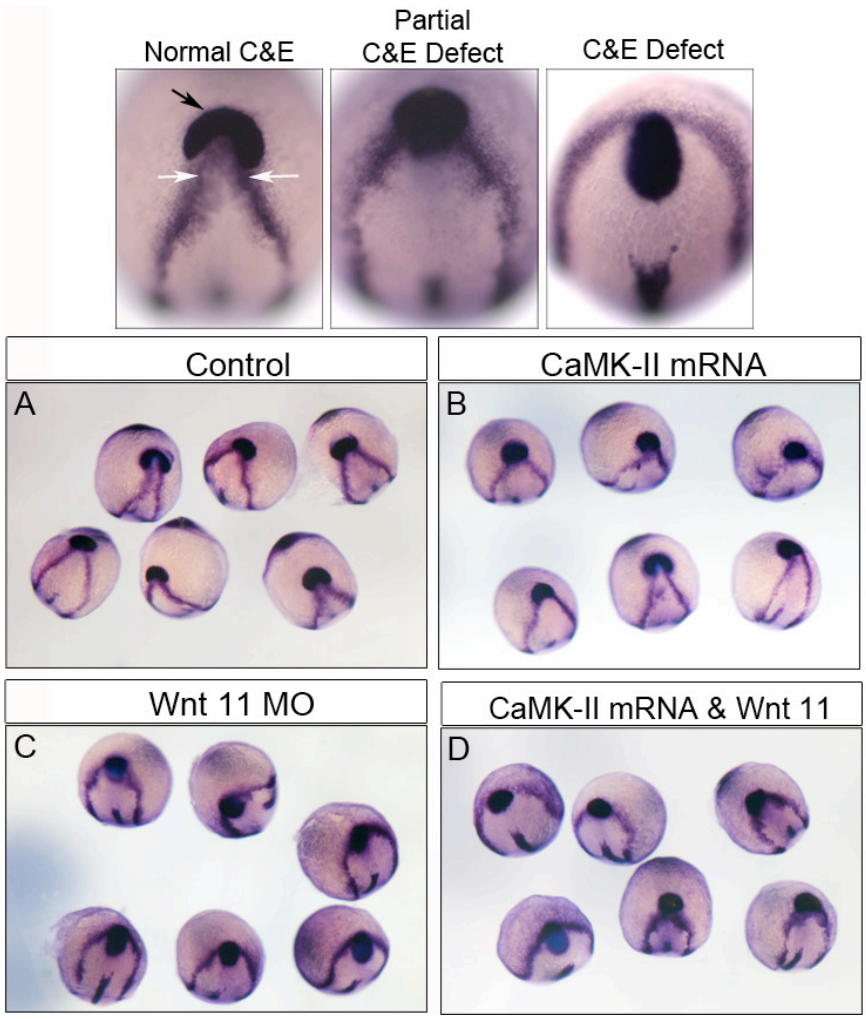

Individual Paraxial Mesoderm Cell Roundness
Cell Roundness
Cell Number
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
20
40
60
80
100
Control
camk2b1 MO
camk2g1 MO

Figure 3-5. CaMK-II is essential for the elongation of paraxial mesoderm cells during convergent extension. (A&B) Axial and paraxial cells of transgenic GFP-CAAX zebrafish embryos were imaged from control and a representative CaMK-II morphant. White arrows and arrowheads indicate width of axial tissue. (C) DIC images of bud stage paraxial cells were used to measure cell roundness. Cells within 6 cell diameters of the notochord were chosen for measurement. Average roundness was calculated from 3 embryos per condition, with a total of 66-86 cells measured per condition.


Normal C&E
Partial C&E Defect
C&E Defect
Control
CaMK-II mRNA
A
B
Wnt 11 MO
CaMK-II mRNA & Wnt 11
C
D

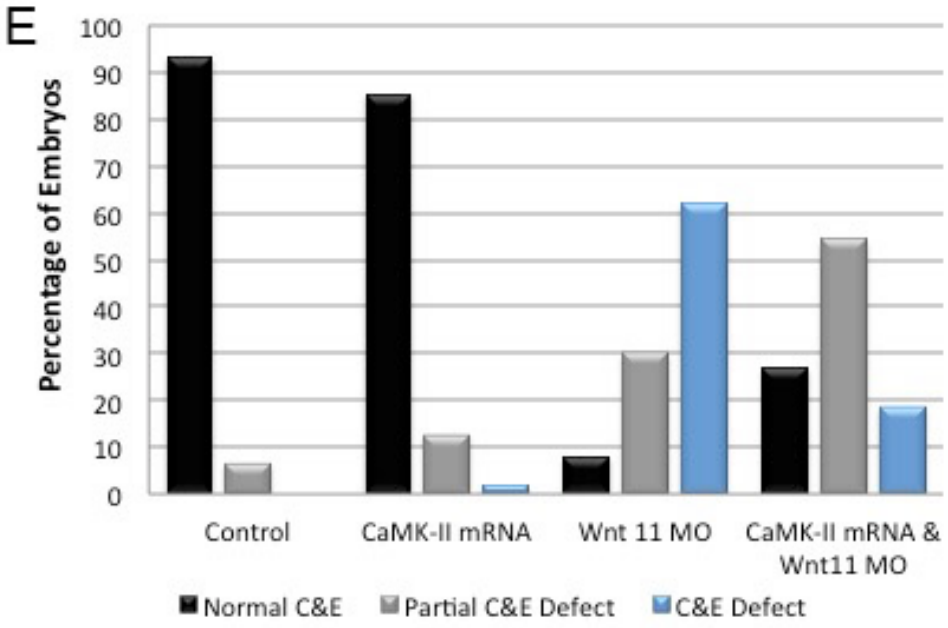

E
Percentage of Embryos
100
90
80
70
60
50
40
30
20
10
0
Control
CaMK-II mRNA
Wnt 11 MO
CaMK-II mRNA & Wnt11 MO
Normal C&E
Partial C&E Defect
C&E Defect

Figure 3-6. **Overexpression of CaMK-II partially rescues the C&E defect of Wnt11 morphants.** *In situ* hybridization with a combination of digoxigenein-labeled RNA probes *dlx3, hgg1* and *ntl* was used to determine the presence of C&E defects. The top three embryos provide a key for classifying C&E defects. Normal C&E is defined by embryos expressing *hgg1* anterior to *dlx3* expression (black arrow) and converged *dlx3* expression (white arrows). Partial C&E embryo defects as classified as having *hgg1* expression in the middle of *dlx3* expression. Finally, C&E defective embryos expression *hgg1* posterior to *dlx3* with no convergence of *dlx3* expression. (A-D) Depict a representative phenotypic group for the control, CaMK-II mRNA, Wnt11 and CaMK-II & Wnt11 conditions injected embryos (E) Quantification of the percentage of embryos from each condition scored for normal C&E, partial C&E defect or C&E defect. 55-107 embryos were counted per condition

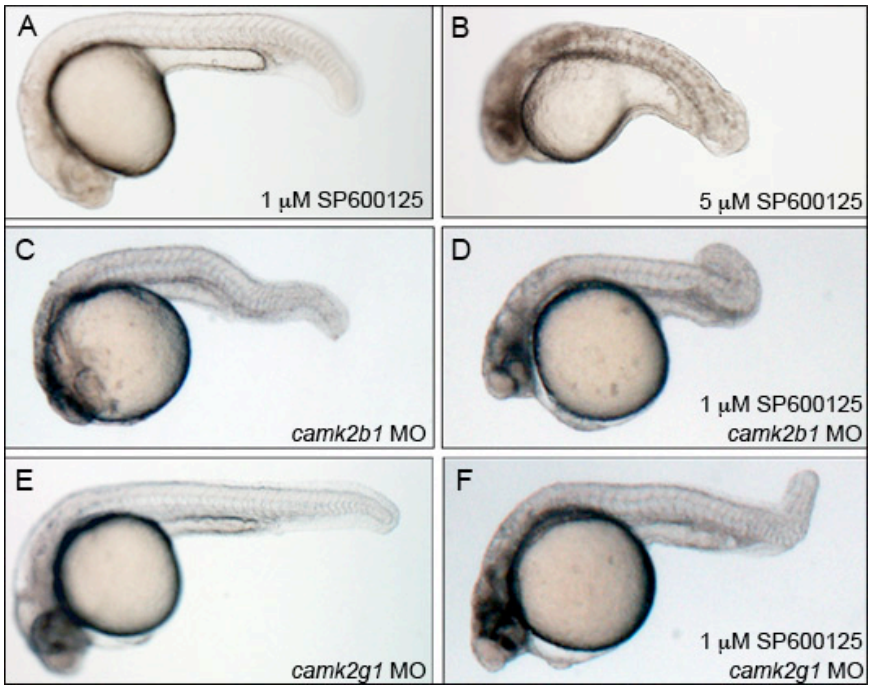


Figure 3-7. **CaMK-II does not act synergistically with JNK to facilitate C&E during zebrafish gastrulation.** (A-F) DIC images of 24hpf embryos treated with JNK inhibitor (SP600125), injected with CaMK-II morpholino or combined SP600125 & CaMK-II treated embryos. Lateral view, anterior to the left.

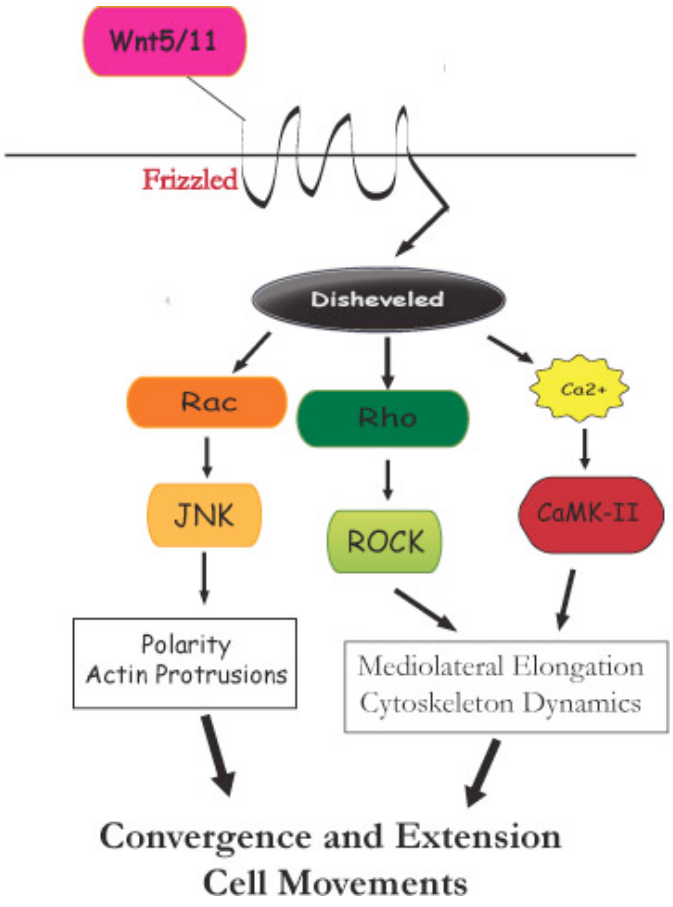


Figure 3-8. Molecular regulation of C&E movements during zebrafish gastrulation

Chapter 4: Final Summary and Perspectives

This project shows that the CaMK-II genes, *camk2b1* and *camk2g1,* are essential for the proper convergence and extension of cells to form the notochord, somites, trunk and trail regions of the zebrafish. We further present evidence that CaMK-II is required specifically for cell elongation and the regulation of cytoskeleton dynamics to facilitate these C&E movements. CaMK-II's regulation on C&E movements is occurring downstream non-canonical Wnt signaling and independently of JNK signaling. In summary our findings identify CaMK-II as an integral protein in facilitating C&E cell movements during gastrulation.

Convergent Extension at the Cellular and Molecular Levels

Bone morphogenetic proteins (Bmp's) are required for the specification of ventrolateral cell fates during embryonic dorsoventral patterning, in addition to specifying the domains for the degree of convergent extension cell movements (Hardt et al., 2007; Myers et al., 2002a). Ventral cells express high Bmp activity, experience 'no C&E' and solely migrate vegetally. Highly converging lateral cell populations move dorsally toward lower Bmp levels, with increasing speeds as they reach the lowest levels of Bmp activity near the dorsal midline (Myers et al., 2002a; Sepich et al., 2000). The results presented in this work show that a knockdown in CaMK-II results in C&E defects that are not due to a change in cell fate. Specifically, the gradient of Bmp morphogen is unaltered in CaMK-II morphants and thus a knockdown in CaMK-II is not altering the gradient of signal required for establishing the domains of C&E, which facilitate the degree of cellular C&E movements.

In addition to a Bmp gradient, regions of rhythmic Ca^{2+} transients have been reported

to occur by 65% epiboly and continue through yolk bud closure (Gilland et al., 1999). These Ca^{2+} waves have been suggested as providing spatial reference for cells, helping to facilitate dorsal-ward cell migration necessary for embryonic axis formation (S. E. Webb and A. L. Miller, 2003). Specifically, Ca^{2+} waves have been linked to cell convergence and extension movements during vertebrate gastrulation; correlating both spatially and temporally with C&E. When Ca^{2+} was blocked pharmacologically, C&E was inhibited without affecting the specification of cell fate (Wallingford et al., 2001). In addition, Ca^{2+} has been identified as a second messenger in the ncWnt pathway (Huelsken and Behrens, 2002; Kühl et al., 2000b; Westfall, 2003; Westfall et al., 2003). ncWnts, Wnt5 and Wnt11, are each capable of activating Ca^{2+} signaling, and both are required for the convergent extension movements of zebrafish gastrulation. Overexpression of ncWnts has been shown to increase intracellular Ca^{2+} release and activate CaMK-II. These Ca^{2+} elevations were only detected during overexpression of ncWnts, with no Ca^{2+} fluxes detected during canonical Wnt overexpression. The work presented in this paper links the activation of CaMK-II, downstream of cytosolic Ca^{2+} fluxes, with C&E movements of zebrafish gastrulation and acting downstream non-canonical Wnt activation. We show that when cytosolic Ca^{2+} was increased pharmacologically, CaMK-II becomes activated and C&E defects are detected. Similar defects were detected during a knockdown in CaMK-II genes, *camk2b1* and *camk2g1.* These results are consistent with ncWnts role in C&E; where either knockdown or overexpression results in similar C&E defects (Wallingford et al., 2000). In addition, we show that overexpression of CaMK-II in Wnt11 morphants is capable of partially rescuing C&E defects; with previous studies identifying similar results when CaMK-II was overexpressed in Wnt5 mutants (Westfall, 2003). Taken together, these results identify

CaMK-II as a target of the ncWnt pathway during zebrafish gastrulation; necessary for regulating C&E cell migrations.

Mediolateral cell movements are the driving force of C&E (Keller et al., 2000). Laterally positioned cells within the gastrula must migrate to the dorsal side; dorsally located cells will then mediolaterally intercalate with adjacent cells to facilitate the formation and extension of the embryonic axis. In each instance, cells must acquire an elongated dorsal-ventral shape. The Rho small GTPase protein family has recently been identified as downstream transducers of ncWnt signaling, necessary in vertebrate mediolateral intercalation. Inhibition or activation of Rho GTPases disrupts gastrulation, impairing cell intercalation of the midline mesoderm (Tahinci and Symes, 2003). Specifically, dishevelled activation, downstream ncWnt activation, leads to independent activation of Rho and Rac (Habas et al., 2003; 2001). In *Xenopus,* researchers have shown that dominant negative Rho and Rac both experience midline tissue extension defects which can only be rescued by overexpression of Rho and Rac, respectively, demonstrating their independent mechanisms for affecting C&E (Tahinci and Symes, 2003). The inhibition of Rho, and not Rac, was shown to prevent cells from acquiring an elongated shape during *Xenopus* gastrulation (Tahinci and Symes, 2003). Historically Rac, and its primary downstream effector c-jun N-terminal kinase (JNK), have been shown to be involved in lamellipodia formation, cell adhesion and cell migration (Sugihara et al., 1998). Based on previous work in mice and *Xenopus,* Rac's role in regulating C&E is suggested to occur through protrusive polarity and lamellipodia extensions (Sugihara et al., 1998; Tahinci and Symes, 2003) (Figure 3-8).

Rho, and its primary downstream effector Rho-assoicated kinase (ROCK), have

previously been shown to be important in cell movements by regulating cell shape and cytoskeleton protrusions (Tahinci and Symes, 2003). Specifically, inhibition of ROCK has been shown to mediate cell polarity and motility through changes in cell shape during zebrafish gastrulation (Marlow et al., 2002). It has been suggested that Rho/ROCK may regulate cell shape changes during gastrulation by modulation of cell-cell contacts and/or destabilization of the cytoskeleton. Rho facilitates cadherin-dependent cell-cell contacts to control cell shape of the ectoderm or mesoderm (S. H. Kim et al., 1998; Wünnenberg-Stapleton et al., 1999; Zhong et al., 1999). Alternately, Rho may be controlling contractility of the cell body by forming stress fibers. When Rho activity is blocked, stress fibers do not form and cells become rounded (Adamson et al., 2002; Kanthou and Tozer, 2002).

Based on the present findings, a knockdown in CaMK-II results in clear disruption of cell morphology at the onset of gastrulation. Individual cell morphology is disrupted within all three germ layers, with cells experiencing a significantly more round morphology and a loss in their ability to elongate medially and laterally. CaMK-II activation during gastrulation has been shown to occur downstream ncWnt and Dsh signaling (Sheldahl, 2003), as was shown with Rho GTPases (T. J. T. Park et al., 2005). To determine if CaMK-II may be working synergistically with Rho GTPases in altering cell morphology during gastrulation, synergism experiments were performed. JNK was inhibited pharmacologically during zebrafish gastrulation, with clear C&E defects detected at 24hpf. When combining JNK inhibitor with CaMK-II morphants, both at concentrations below the threshold for significant C&E defects, no additional defects were detected suggesting that CaMK-II and Rac/JNK are not working synergistically to facilitate C&E cell movements. To determine if CaMK-II may be working synergistically with Rok signaling, inhibition of Rok

was attempted via pharmacological treatment. Unfortunately, the results of this experiment were inconclusive, with high concentrations of inhibitor alone unable to cause the C&E defects, which had previously been reported. Continued experiments are being designed, using ROCK morpholino and/or Rho inhibitor C3-exoenzyme. However, based on the literature, we believe Rho and CaMK-II are working synergistically. Inhibition of Rok results in a similar rounded cell morphology, as seen during a knockdown in CaMK-II (Tahinci and Symes, 2003). In addition, Rho activity is specifically connected to cell contractility (Adamson et al., 2002; Kanthou and Tozer, 2002) which also appears disrupted in CaMK-II morphants, as displayed by a circular, instead of oblong, yolk-cell at early somitogenesis.

Potential downstream effectors of CaMK-II, regulating cell shape and migration to alter C&E motility during gastrulation include the focal adhesion protein, focal adhesion kinase (FAK). CaMK-II has been shown previously to regulate focal adhesion turnover during cell motility via FAK regulation; inhibition of CaMK-II resulted in over-stabilized focal adhesions with increased and frozen lamellipodia dynamics and constitutive activation of CaMK-II leading to rounded cell morphology and a decrease in phosphorylated FAK (Easley et al., 2008). *Xenopus* research has shown that FAK activation increases significantly throughout the mesoderm and ectoderm during gastrulation (Hens and DeSimone, 1995), with zebrafish research showing an increase in FAK activation at the somite boundaries suggesting a role in boundary formation (Crawford et al., 2003; Henry et al., 2001). An additional potential target of CaMK-II, facilitating cell morphology changes during C&E, is non-muscle myosin. Non-muscle myosin has been identified as a substrate of CaMK-II phosphorylation (Buxton and Adelstein, 2000). When non-muscle myosin II

activity is impaired, the cortical actin network is disrupted, cells fail to exhibit normal protrusive activity, and C&E movements are reduced (Paluch and C.-P. Heisenberg, 2009; Skoglund et al., 2008). In addition, interfering with myosin activity also affects cell properties other than contractility, such as cell adhesion (Paluch and C.-P. Heisenberg, 2009; Skoglund et al., 2008). Together, FAK and non-muscle myosin II are two potential targets of CaMK-II to facilitate C&E movements during gastrulation, with continued research necessary to test our hypothesis.

Interestingly, both focal adhesion and myosin II regulation have also been proposed as downstream effectors of Rho/ROCK, further suggesting the potential for synergy. ROCK has previously been shown to induce the formation of focal adhesions and assembly of stress fibers necessary for active migration (Ridley and Hall, 1992). In addition, ROCK has been shown to phosphorylate non-muslce myson II regulatory light chain (MRLC) to restrict actin bundle formation and again facilitate cell motility (Zhu et al., 2006). Taken together FAK and non-muscle myosin II are two potential targets of CaMK-II in regulating cell morphology and migration during gastrulation, in addition to working synergistically with ROCK to mediate these effects.

CaMK-II: A Multifunctional Protein Family

CaMK-II is a highly conserved Ca^{2+}/calmodulin-dependent protein kinase expressed throughout the lifespan of vertebrates. Seven transcriptionally active genes encoding CaMK-II have been described in zebrafish embryos including: *camk2a1, camk2b1, camk2b2, camk2g1, camk2g2, camk2d1* and *camk2b1* (Rothschild et al., 2007). Upon identifying the differential expression pattern each gene has during early zebrafish embryogenesis,

continued characterization of each genes function has been explored. To determine CaMK-II's role in zebrafish development, gene specific morpholinos were designed and the effects associated with each morpholino have been characterized. With CaMK-II known to account for 2% of the total protein in a vertebrate brain (Erondu and Kennedy, 1985), primary focus has been directed towards identifying non-neuronal effects that make CaMK-II developmentally essential. Of the seven genes identified, morpholinos against *camk2a1, camk2b1, camk2b2,* and *camk2g1* have all been shown to cause unique and specific effects, with *camk2d1* and *camk2d2* only characterized as showing brain defects thus far.

Specifically, CaMK-II has been shown to play a role in proper heart, fin and kidney development, as well as establishing embryonic L-R asymmetry. For each of these developmental processes, the expression and activity of specific CaMK-II genes is necessary. *Camk2b2,* and not its paralog *camk2b1,* is necessary for regulating heart and fin development (Rothschild et al., 2009), *camk2g1* for kidney development (Rothschild et al., 2011), and *camk2a1* (αKap), *camk2b2* and *camk2g1* expression and activation in regulating L-R asymmetry (Francescatto et al., 2010). The results presented in this paper find that *camk2b1* and *camk2g1* are necessary for C&E during zebrafish gastrulation, and demonstrate the specificity of each gene during this process, with no synergistic affects detected during simultaneous knockdown. Ongoing research in the lab hopes to determine additional role for each gene in development.

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