REGULATION OF CELL MOVEMENTS BY CHEMOKINE APELIN DURING ZEBRAFISH DEVELOPMENT

By

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ABSTRACT

Proper embryonic development requires precise cell movements, which are coordinated by multiple signaling pathways. Formation of specific organs is initiated during gastrulation when organ precursors acquire their initial cell fates. Additionally, precursors move to specific locations where they engage in additional inductive interactions to continue their differentiation, and form the specialized tissues required for organ functions. Impaired cell movements can cause severe embryonic malformation, developmental arrest and also many related diseases. In this work, I used the zebrafish, *Danio rerio*, a well-established vertebrate model system to study the involvement of G-protein coupled receptor (GPCR) signaling in cell movements.

Apelin and its GPCR receptor Agtrl1 regulate adult physiology, in particular cardiovascular functions, and blood vessel development. Here we show that the zebrafish Apelin and Agtrl1b homologs control heart field formation during gastrulation. Cardiac precursors, specified in the lateral plate mesoderm territories, converge toward the embryonic midline during gastrulation and extend rostrally to form bilateral heart fields. We found that *agtrl1b* is expressed in the forming mesendoderm before gastrulation, and in the lateral plate mesoderm later, while *apelin* expression is confined to the midline. Suppressing the function of Agtrl1b or its ligand Apelin using morpholino antisense oligonucleotides resulted in a deficiency of cardiac precursors and a subsequent absence or reduction of heart. Embryos injected with *apelin* RNA formed no heart. Our cell tracing experiments demonstrated that in embryos with excess Apelin, cardiac precursors failed to move to the correct location and to express heart markers. Time-lapse analyses

of Apelin overexpressing gastrulae revealed reduced migration and defective morphology of mesodermal cells including cardiac precursors. Moreover, in Apelin deficient gastrulae, the cardiac precursors moved less efficiently to the correct location, and showed broadened and ectopic distribution. Our results demonstrate an essential developmental role for the Apelin-Agtrl1b GPCR signaling system in mesodermal cell movements and migration of cardiac precursors to form the heart field during vertebrate gastrulation.

During our investigation, we found that Apelin also regulates the migration of zebrafish primordial germ cells (PGCs), a process previously shown to be regulated by Sdf1a/Cxcr4b GPCR signaling. During gastrulation and somitogenesis, *apelin* mRNA is expressed in the dorsal midline, while its receptor *agtrl1b* gene is broadly expressed in the mesendoderm, where PGCs are localized. Manipulating Apelin function by misexpression throughout the embryo or by overexpression specifically in primordial germ cells impaired movements of PGCs towards their target tissues. Suppressing Apelin function by injections of antisense morpholino oligonucleotides also resulted in a phenotype of mis-localized PGCs. The abnormal PGC movements in these loss and gain of function scenarios are not a consequence of altered *sdf1a* expression. Using transplantation experiments, we showed that the cells expressing Apelin in ectopic locations attracted PGCs. However, in these experiments the PGCs stopped short of Apelin overexpressing cells. Interaction between both Apelin and Sdf1a signaling was also investigated. In *odysseus* (*ody*) (-/-) mutants, which harbor a null mutation in *cxcr4b* gene, the majority of ectopic PGCs aggregate in the dorsal midline where *apelin* is expressed. Interference with both signaling pathways, by injecting *apelin* MO into *ody* (-

/-) mutant embryos, significantly reduced the dorsal aggregation of PGCs. Based on the preliminary data, I hypothesize that Apelin provides an attractive cue for PGCs migration during gastrulation and segmentation stages, in addition to the previously discovered Sdf1a/Cxcr4b signaling pathway.

To my parents, Guangzhan Zeng and Songlian He, for constant support and love.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

μg	microgram
μm	micrometer
A	Animal pole
AP	Anterior-Posterior
Agtrl1a	Angiotensin II receptor-like 1a
Agtrl1b	Angiotensin II receptor-like 1b
atgMO	Translation-blocking mopholino
bp	base pair
C&E	Convergence and Extension
cmlc2	*cardiac myosin light chain 2*
D	Dorsal
dpf	Days Post-Fertilization
DV	Dorsal-Ventral
ENU	N-ethyl N-nitrosourea
G Protein	Guanine nucleotide exchange protein
GFP	Green fluorescent protein
GPCR	G-Protein coupled receptor
hpf	Hours Post-Fertilization
LPM	Lateral Plate Mesoderm
LWR	Length-to-width ratio

ML	Mediolateral
MO	Morpholino Oligonucleotides
MO^{atg}	Translation-blocking mopholino
MO^{splice}	mRNA Splicing-interfering mopholino
nos1	*nanos1*
NC	notochord
ng	nanogram
ntl	*no tail*
PBS	Phosphate buffered saline
PCP	Planar Cell Polarity
PH	Pleckstrin-Homology
pg	picogram
PKC	Protein kinase C
RNA	ribonucleatic acid
Stat3	Signal transducer and activator of transcription 3
TB	Tailbud
TILLLING	Targeting Local Lesion IN Genomes
Wnt	Wingless
WT	Wild-Type
UAS	Upstream Activating Sequence
V	vegetal pole
YSL	York syncytial layer

CHAPTER I

INTRODUCTION

Cell movement

Cell movement is a critical process for every type of animal organism. Single-celled organisms use cell movement as a key survival strategy for seeking food, avoiding predators and mating (Manahan et al., 2004). In metazoans, cells will often move from one location to another to undergo differentiation, form specialized organs, or carry out functions. Tissue formation during embryonic development, wound healing and immune responses all require the accurately coordinated movement of cells in a particular direction and to a specific location (Ridley et al., 2003). Understanding cell movement is also an important part of cancer research. One of the key differences between a benign and malignant cancer is the ability to metastasize, in which malignant cells migrate from the original tumor toward many other tissues (Sahai, 2007). Therefore, a greater understanding of how cells migrate in normal development could result in new therapies for cancer.

Cell movements occur at many levels of organization. Our current knowledge could divide directed cell movements into three main categories in terms of scale: *movement of individual cells*, such as in bacteria, *Dictyostelllium amoebae*, fibroblasts in culture and neuronal migration; *movement of cell groups*, such as border cells in *D. melanogaster*, primordial germ cells in zebrafish and

directed migration of mesodermal cell populations during zebrafish gastrulation; *movements of cell sheets*, such as dorsal closure in *D. melanogaster*, wound healing and involution during gastrulation in *X. laevis*. It is also worth to mention that movements of cell sheets often involve cell rearrangements that lead to tissue morphogenesis (e.g., mediolateral intercalation or radial intercalation).

During the work of this dissertation, I mainly focused on cell movements during vertebrate embryonic development. The establishment of the embryonic architecture, the patterning formation while involved cell fate specification, acquires a tremendous participation of cell movements.

All vertebrate organisms began life as a single, genetically complete cell (the zygote, or fertilized egg), resulting from the union of a sperm and egg. After conception, the zygote divides rapidly to form a mass of cells. Large groups of these dividing cells subsequently engage in gastrulation, a morphogenetic process that generates and shapes the germ layers. During gastrulation, the presumptive mesoderm and endoderm move inside the ectoderm, to form multiple-germ layers consisting of ectoderm outside, endoderm inside, and mesoderm in between (and see below). Following gastrulation, cells within these layers continue to move to find their target destinations in the developing embryo and to differentiate into components of specialized organs. In the developing brain, for example, neuronal cells migrate within the neural tube, where they send projections (axons and dendrites) through the layers of developing cells to their final targets to form specific synaptic connections, which mediate complex functions such as learning and memory.

Within vertebrate embryos, there are two types of cell populations based on their different properties and organizations: epithelial and mesenchymal cells. First, epithelial cells are tightly packed together with little intercellular space, and various types of tight cell junctions present between the apical and basal domains to facilitate the formation of the continuous cell layer. Individual epithelial cells show a lack of mobility with respect to their local environment (Larue and Bellacosa, 2005). Epithelial cells connected via strong cell adhesion move as integrated tissue sheets in a coordinated manner. A good example could be the process of zebrafish ectodermal cell movements during gastrulation (Concha and Adams, 1998). However, epithelial cell movements do involve a dynamical breaking down and re-establishing of cell junctions, such as during epiboly movement of enveloping layer (and see below). Second, mesenchymal cells without clear apical and lateral membranes can migrate as individual or small groups using loose or no interaction among cells, as seen in the neural crest migration and primordial germ cell migration in all vertebrate animals (Larue and Bellacosa, 2005). My work described in this dissertation will focus on movements of mesenchymal cells.

During the last several decades, scientists have been intensively studying cell movements in many different model systems, and trying to understand following important questions: which types of molecules are required in cell movements and what are their roles in signaling pathways? What is the mechanism of the cell motility? How is motility regulated? What is the mechanism of directed cell migration?

Our knowledge about the mechanism of cell migration is mostly based on a classical model of cell migration, an individual fibroblast-like cell migrating on a 2D environment. A migrating cell is a polarized cell with distinct leading and trailing edges. The leading edge points in the direction of movement and is driven by actin-polymerization-mediated protrusion. During migration, cell adhesions assemble at the leading edge and disassemble at the trailing edge (Vicente-Manzanares et al., 2005). There is a nascent but increasing literature on cells migrating in 3D environments or *in vivo* condition, but little is known about the molecular details (Beningo et al., 2004; Gunzer et al., 2000).

Our group uses zebrafish as a model system to study vertebrate gastrulation process, especially mesodemal cell movements and also associated mechanisms.

Zebrafish as a model system to study cell movement

The zebrafish, *Danio rerio*, is a tropical fish belonging to the minnow family (*Cyprinidae*). The benefits of the zebrafish model system for genetic and developmental studies include a short reproductive cycle of 2-3 months, external fertilization, and a transparent embryo, affording detailed microscopic inspection of the embryo throughout all phases of development.

The pioneering work of George Streisinger (University of Oregon) first introduced the zebrafish as a model organism. The milestone event consolidating its importance were two significant large-scale forward genetic screens for *N*-ethyl-*N*-nitrosourea (ENU) induced embryonic lethal mutations. These screens

are commonly referred to as the Tübingen/Boston screens, in which researchers identified hundreds of zebrafish mutations affecting early development and organogenesis. The outcome of these screenings led to a special issue of journal Development (Zebrafish Issue, Development 123, 1996), introducing mutations affecting body patterning, cell movement, neurogenesis, and organ development. Also, construction of genetic maps and progress made in the zebrafish genome sequencing has made zebrafish study feasible at the molecular level. An online database of zebrafish genetic, genomic, and developmental information (ZFIN) has been established. More recently, methods for inactivation of a specific gene's function in zebrafish system became feasible. Antisense Morpholino oligonucleotides (MOs) (reverse genetics) are synthetic molecules with a modified nucleic acid structure (Summerton and Weller, 1997). Their completely unnatural backbones protect MOs from recognition and degradation by cellular nucleases (Hudziak et al., 1996). One could inject MO into zebrafish embryo zygote to interfere with the RNA translation or splicing process of a specific gene, and consequently result in the loss of function in this protein (Nasevicius and Ekker, 2000). Originally applied in plant research, TILLING (Targeting Induced Local Lesions IN Genomes) (reverse genetics) is a new approach that allows one to identify ENU-induced mutations in known genes and has been successfully applied in zebrafish (Hurlstone et al., 2003; Wienholds et al., 2003). Another important advantage for the optically transparent zebrafish is the generation of transgenic zebrafish lines, which use regulatory DNA to drive expression of fluorescent proteins reporters in temporally and spatially regulated patterns allow

visualization of cells in developing embryos (Deiters, 2006). Application of this technology in zebrafish enables visual analysis of regulation of gene expression in a living organism easily, and it is highly valuable for achieving sophisticated cell fate-mapping and other associated analyses. For example, *Tg(flk1:egfp)*^{s843} has been used broadly for studying the development of vascular system (Jin et al., 2005). *Tg(olig2:egfp)* (Shin et al., 2003) and *Tg(nkx2.2a:megfp)* (Ng et al., 2005) have been used to investigate the dynamic oligodendrocyte progenitor behavior during zebrafish development (Kirby et al., 2006). All of these forward and reverse genetic analyses combined with embryological and molecular methods make the investigation of genetic mechanisms underlying cell specification and migration during gastrulation and organogenesis particularly effective in zebrafish.

Zebrafish gastrulation movements and underlying signaling pathways

Gastrulation is a crucial period early in the development of multi-cellular organisms during which the morphology of the embryo is dramatically restructured by cell movements and rearrangements. It involves the mass movement of cells to form complex structures from a simple starting form. These global changes in form are achieved by four morphogenetic movements during zebrafish gastrula period: epiboly, internalization, convergence and extension movements (Figure 1). Concurrent with all of these movements, cells within the embryo acquire different fates patterning anteroposterior and dorsoventral asymmetries into the body rudiment.

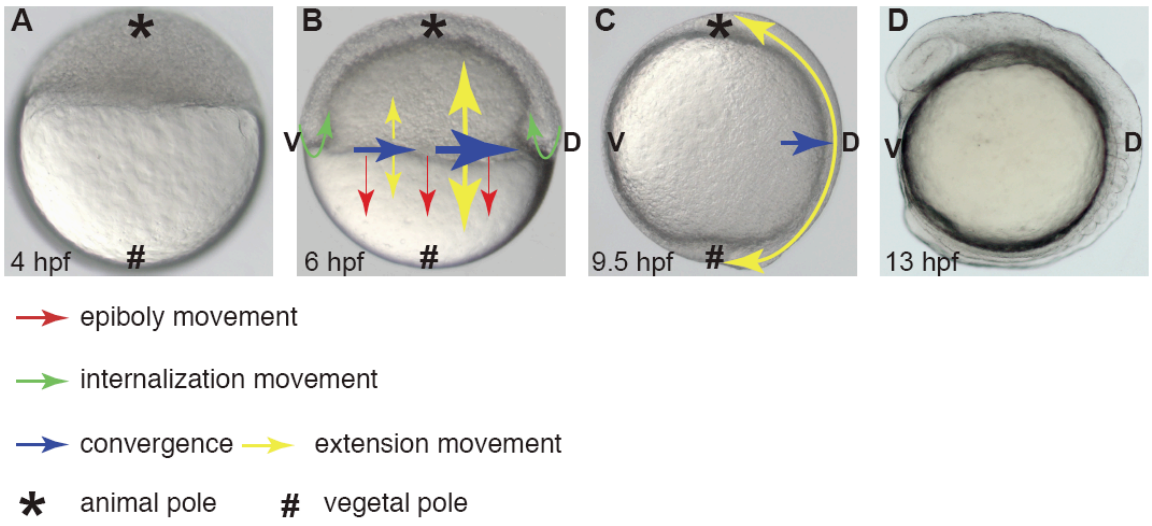

Figure 1. Gastrulation cell movements.
Photographs of live zebrafish embryos at the indicated developmental stages. Green lines represent internalization; red lines represent epiboly; blue lines represent convergence; yellow lines represent extension. Star, animal pole; #, vegetal pole; V, ventral; D, dorsal.

At 4 hours post-fertilization (hpf), when the zygotic genome is activated (Kane and Kimmel, 1993), the zebrafish embryo exhibits a symmetrical morphology along the anteroposterior axis; it consists of around 1000 blastomeres sitting on the top of a large yolk cell (Figure 1A). At this stage three distinct cell layers can be visualized. The enveloping layer (EVL) has formed a thin superficial sheet on the outside of the blastomeres. The yolk syncytial layer (YSL) is formed from the fusion of cells (or blastomeres) adjacent to the yolk cell. The deep cells giving rise to all embryonic tissues are sandwiched between by EVL and YSL. Epiboly movement is the first morphogenetic movement during zebrafish gastrulation (Warga and Kimmel, 1990) (Figure 1B). Leading to vegetal expansion and thinning of the blastoderm, epiboly occurs in the deep cells over the YSL, and is driven by the migration of nuclei and cytoplasm in the YSL as well as attachments between the YSL and the EVL (Wilson et al., 1995). Epiboly ends when the blastoderm covers the entire yolk at the end of the gastrula period (Figure 1C).

In zebrafish, endoderm precursors are derived from cells close to the yolk syncytial layer (YSL) at the blastoderm margin (40% epiboly stage) (Schier and Shen, 2000). At the beginning of gastrulation, ectoderm precursors in zebrafish blastula are localized at the animal pole that is the upper hemisphere consisting of rapidly dividing cells in the embryo, and they are above the prospective mesoderm (Shen and Schier, 2000) (Figure 1A). More specifically, neural ectodermal tissues and axial mesoderm are concentrated in the dorsal region, and non-neural ectoderm and other ventroposterior mesoderm are positioned at

the ventral side of the gastrula (Helde et al., 1994).

At 5.5 hpf, when the gastrula period begins, zebrafish embryos acquire a sphere-like shape, still with a symmetric morphology as viewed from the animal pole. Prospective mesodemal and endodermal cells at the blastodermal margin start to undergo internalization. In this process the presumptive mesoderm or endoderm moves as individuals or cell groups from the outer layer of the gastrula into the inner and deeper layer to underlie the ectoderm (Solnica-Krezel, 2006) (Figure 1B). Less than 30 minutes later, the embryonic shield in the dorsal side starts to form by a larger thickening (Figure 1B). The zebrafish embryonic shield, equivalent to the Spemann-Mangold organizer in Xenopus, is the key embryonic signaling center to direct gastrulation movement, induce axis formation and specify tissue fate (Bouwmeester, 2001; Niehrs, 2005; Sander and Faessler, 2001; Solnica-Krezel, 2006).

During zebrafish gastrulation, Convergence and Extension (C&E) cell movements drive the overall process of mediolateral narrowing and anteroposterior elongation of embryonic tissues (Solnica-Krezel and Cooper, 2002). All germ layers undergo C&E movements, and I will mainly focus on the mesoderm layer in this dissertation. During early gastrulation, upon internalization, mesendodermal cells migrate individually to contribute to the elongation of the mesoderm by moving toward the animal pole, without dorsal movement (Sepich et al., 2005). The developmental fate of mesoderm varies along the dorsal to ventral axis bilaterally, including the axial, paraxial, intermediate, and lateral mesoderm (Kimmel et al., 1990). Different types of

mesodermal cells use different cell behaviors during C&E movement at later stages of gastrulation (Keller et al., 2000; Sepich et al., 2005; Sepich and Solnica-Krezel, 2005; Solnica-Krezel and Cooper, 2002). I am going to focus on discussing lateral mesoderm during C&E. Lateral mesodermal cells start to convergence toward the dorsal side of the embryo starts at 70% epiboly stage (7.7 hpf), and exhibit directional preference, directionally-regulated speed and turn toward the dorsal side when off-course (Sepich et al., 2005). This interesting observation lead to a hypothesis that these lateral mesodermal cells moving toward dorsal region might migrate toward chemoattractants distributed along the dorsal midline via the mechanism of chemotaxis (and see below).

The end result of gastrulation is a polarized three germ-layered embryo (Figure I-C): the ectoderm, the outer layer of the embryonic body plan, is the precursor to the epidermal layer and the nervous system. The mesoderm, the middle layer of the embryonic body plan, is the precursor to the skeleton, muscle, connective tissues, kidneys, circulatory, and reproductive systems. The endoderm eventually forms the inner, epithelial layer of the gut and its associated organs, including the liver and the pancreas. Gastrulation is followed by organogenesis, when individual organs develop within the newly formed germ layers and through their interactions.

Several signaling pathways have been implicated in directing large-scale gastrulation movements in vertebrates, including Stat3, Gα12/13, Prostaglandin E2 - EP4, noncanonical Wnt, and FGF signaling (Keller, 2005; Leptin, 2005; Solnica-Krezel, 2005). Previous studies demonstrated that C&E movements of

lateral mesodermal cells in zebrafish embryos are depended on non-canonical Wnt and Stat3 (Jessen et al., 2002; Yamashita et al., 2002; Yamashita et al., 2004). Hyaluronic acid synthesizing enzyme 2 (Has2) is required for convergence but not extension of lateral mesodermal cells in a cell-autonomous manner, and GTPase Rac1 is believed to be the downstream effector that promotes cell protrusions (Bakkers et al., 2004). Recently, it has been shown that Prostaglandin E2 acts through the EP4 receptor, a member in the G-protein coupled receptor (GPCR) superfamily, to activate Akt at least in part to regulate the motility of converging mesodermal cells (Cha et al., 2006). Zebrafish embryos lacking the function of Gα12 and Gα13 proteins, known regulators of Rho, exhibit rounder cell shapes and change movement direction more frequently than their wild-type siblings in the converging mesodermal cells (Lin et al., 2005). Based on above summarized data, in particularly the involvement of heterotrimeric G proteins and EP4 GPCR, we hypothesized that certain unknown G-protein coupled receptors, upstream regulators of heterotrimeric G-Proteins, regulate zebrafish gastrulation movements, especially the involvement in the convergence movements of these lateral mesodermal cells.

Cell movements during zebrafish heart development

In above paragraphs, I discussed briefly the cell behaviors and signaling pathways involved in migration of lateral mesodemal cells during zebrafish gastrulation. One important and interesting cell population, the cardiac precursor cells, resides within the region of embryonic lateral mesoderm. I will discuss their

movements during zebrafish gastrulation and signaling pathways involved in their regulation in following sections.

During vertebrate organogenesis, the corresponding progenitor cells acquire initial cell fates through the influence of multiple inductive signals existing in the embryo (Auman and Yelon, 2004). They typically form organ primordia by moving toward a specific location, or condensing into an organ rudiment, and later continue differentiation to form the specialized cells or tissues required for organ function. In vertebrates, myocardial progenitor cells can first be distinguished by *nkx2.5* expression in two bilateral stripes of cells in the anterior lateral plate mesoderm (ALPM) at early somitogenesis stages (Harvey, et al., 1996). Fate-mapping studies in several vertebrates have shown that, prior to the onset of *nkx2.5* expression, the heart anlage arises during gastrulation when prospective cardiac precursors, specified in the lateral plate mesoderm territories (Figure 2A), converge toward the embryonic midline and extend rostrally to form bilateral heart fields at late gastrulation (Figure 2B-E) (Keegan et al., 2004; Parameswaran and Tam, 1995; Schoenwolf and Garcia-Martinez, 1995; Stainier et al., 1993). Multiple signaling pathways, acting in both stimulatory and inhibitory fashions, act to restrict cardiomyogenesis to a defined domain in the ALPM. These signals include TGF-βs, Fgfs, Shh, Retinoic Acid, and Wnts/Wnt inhibitors (Keegan et al., 2005; Reifers et al., 2000; Schneider and Mercola, 2001; Schultheiss et al., 1997; Zhang et al., 2001). In zebrafish, lysosphingolipid signaling via the Miles apart GPCR is essential for movements and fusion of the bilateral heart primordia during segmentation (Kupperman et al., 2000). However,

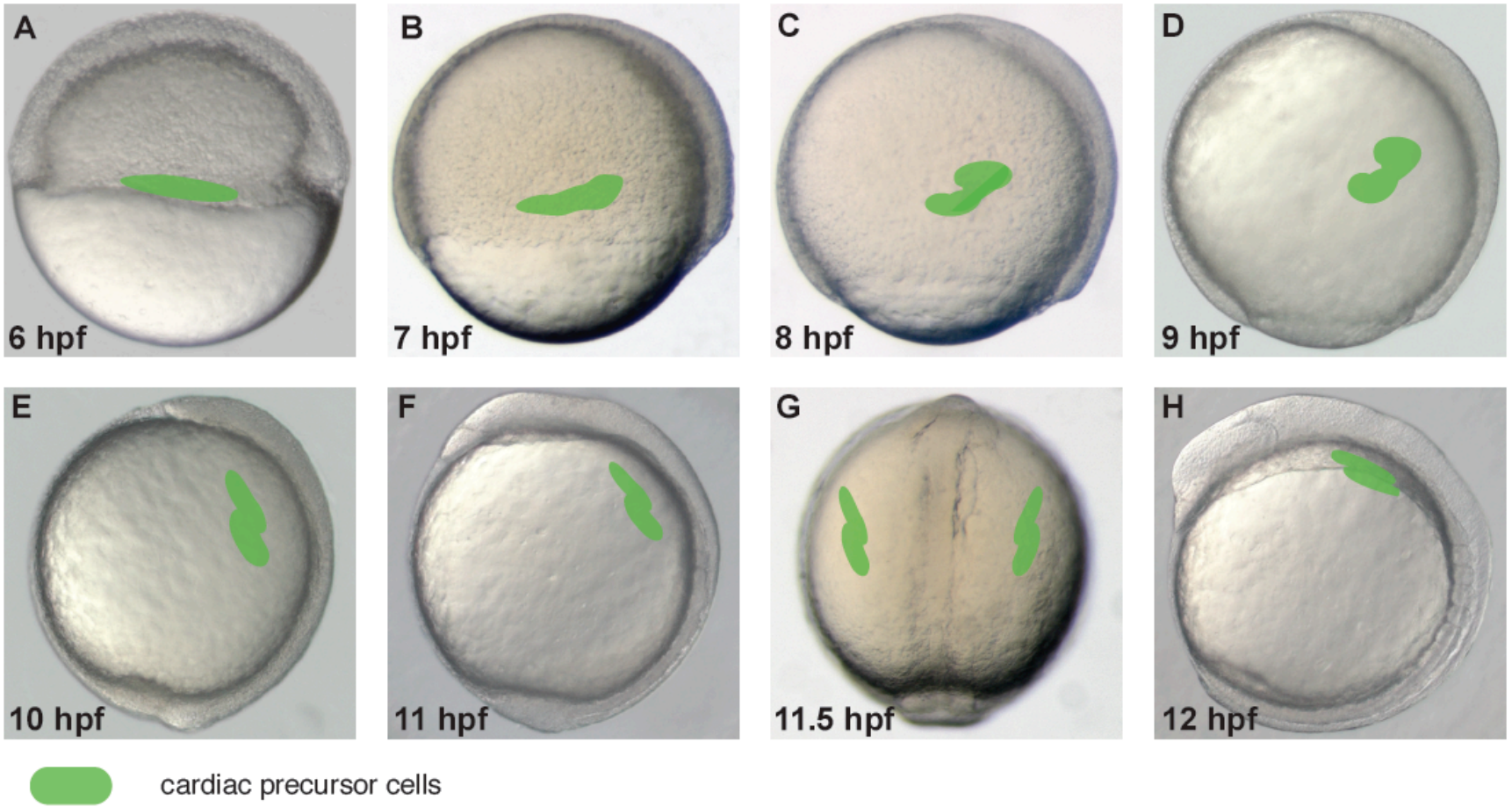

Figure 2. The spatial organization of cardiac progenitors at zebrafish early developmental stages.
(A-H) Schematic representations of zones containing cardiac progenitors (green). (A-F, H) lateral views, dorsal towards the right, at (A) shield, (B) 60% epiboly, (C) 75% epiboly, (D) 90% epiboly, (E) tailbud, (F) 2 somites and (H) 6 somites stages. (G) Dorsal views, anterior towards the top, depicting cardiac progenitor zones within the LPM at 4 somites.

relatively little is known regarding the mechanisms that specify myocardial progenitor and direct their migration to the ALPM prior to the onset of *nkx2.5* expression. Explant studies in chick have suggested that signals are required for cardiomyogenesis prior to gastrulation (Antin et al., 1994). As we introduced before, all of these signaling pathways that affect C&E movements of the three germ layers also consequently secondarily affect the movements of heart progenitors. We ask, during gastrulation before the onset of *nkx2.5* activation, whether other pathways that could regulate movements of the defined cell populations, like the cardiac precursors, also operate during vertebrate gastrulation is an open question.

Primordial germ cells (PGCs)

In my work of this dissertation, we also investigated another type of interesting cell population — the zebrafish primordial germ cells (PGCs). Following sections will discuss their fate specification, migration process and signaling pathways that regulate their migration.

All sexually reproducing organisms start their development from the fusion of sperm and eggs, the gametes that are responsible for transmitting genetic information from one generation to the next. All gametes arise from the PGCs. PGCs have emerged as an excellent model for studying directional cell migration. During development of zebrafish and many other organisms, the PGCs migrate a long distance from the regions where they are specified towards the developing gonad where they generate gametes (Antin et al., 1994; Wylie,

2000). Understanding the mechanisms that guide PGCs as they travel towards their destination concurrent with the morphogenetic gastrulation movements of somatic tissues along the migration path represents a particularly interesting problem of long-distance cell movement during embryogenesis.

The specification of zebrafish PGCs was first documented by the germline-specific marker *vasa* (Olsen et al., 1997; Yoon et al., 1997). This gene was originally identified in Drosophila and encodes an ATP-dependent RNA helicase of the DAD BOX family (Hay et al., 1988). As in several other organisms, the zebrafish *vasa* RNA is maternally supplied and expressed in the germline throughout the entire development. Hence, *vasa* RNA affords PGC recognition and examination from the earliest embryonic stages of development to the later gamete differentiation stages.

In the zebrafish embryo at dome stage (4.3 hpf), the PGCs reside in four positions on the blastoderm margin, randomly located with respect to the prospective embryonic axis (Weidinger et al., 1999). Most migrate toward the dorsal midline, but often, one of the four clusters migrates away from the dorsal region if it is already located in that region at the beginning of migration (Figure 3A). During gastrulation and early somitogenesis stages, the PGCs align along the lateral borders of the trunk mesoderm at the boundary between the head and trunk mesoderm (Figure 3B) (Weidinger et al., 1999). The PGCs then migrate toward two regions flanking the first three somites, and form two bilateral-cell clusters by the 6-to 8-somite stage (Weidinger et al., 1999). Subsequently, the PGCs leave the earlier-formed somites level and migrate to form bilateral

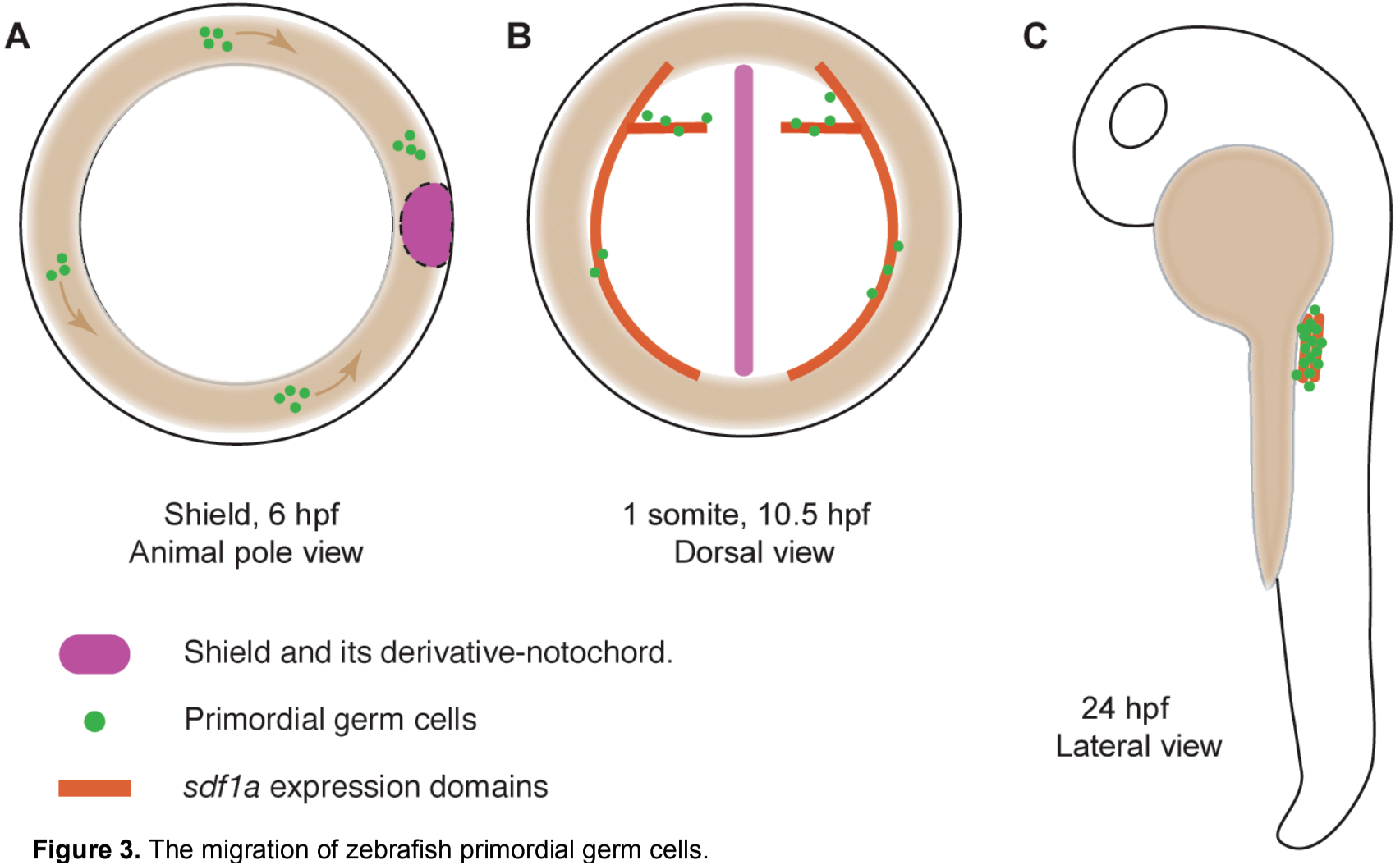

Figure 3. The migration of zebrafish primordial germ cells.

several cell types, such as lymphocytes (Bleul et al., 1996a; Bleul et al., 1996b), cerebellar and hippocampal neurons (Lu et al., 2002; Zou et al., 1998). It also plays a part in several pathological situations, for example, tumor metastasis (Muller et al., 2001) and joint infiltration (Buckley et al., 2000). Recent studies also showed that in mouse, germ cell migration and survival requires the SDF1/CXCR4 interaction (Molyneaux et al., 2003). The fact that germ cell development is affected in CXCR4 mutant mice and zebrafish further suggests that a conserved G-protein-coupled receptor-signaling mechanism regulates early germ cell development in vertebrates.

G-Protein coupled receptors

G-protein coupled receptors (GPCRs), also known as seven transmembrane receptors, 7TM receptors, heptahelical receptors, and G protein linked receptors (GPLR), constitute a large protein family of transmembrane receptors that sense molecules outside the cell and activate the internal signal transduction through heterotrimeric G protein-dependent and -independent pathways and, ultimately, cellular responses (Figure 4). The ligands that bind and activate these receptors include light-sensitive compounds, odorants, pheromones, hormones, and neuro-transmitters, and vary in size from small molecules to peptides to large proteins. These signaling pathways regulate key biological processes such as cell proliferation, cell survival and angiogenesis (Marinissen and Gutkind, 2001). According to the binding ligands, GPCRs are grouped into two classes (Vassilatis et al., 2003). One major group, referred to as

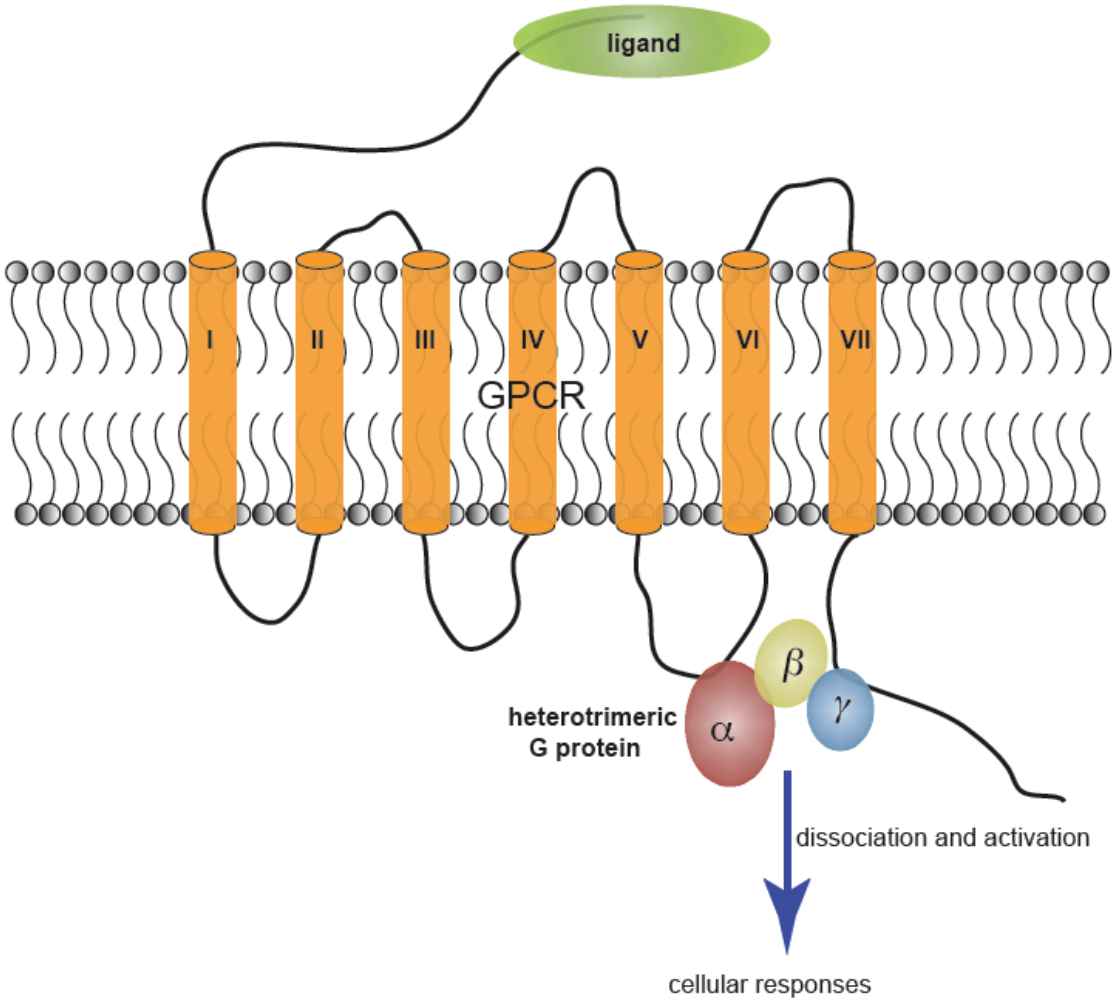

Figure 4. Seven trans-membrane G-Protein Coupled Receptor (GPCR) and other components in the siganling pathway.

clusters at the level of the 10th somite. By the end of the first day of development, all PGCs are found at the positions where the future gonads develop during the following weeks (Figure 3C) (Weidinger et al., 1999).

The signals directing movement of PGCs in vertebrate embryos are beginning to be elucidated. Recent work has identified the chemokine stromal-cell-derived-factor (SDF)-1a as a pivotal secreted signal guiding PGC migration in zebrafish embryos (Doitsidou et al., 2002; Knaut et al., 2003). *sdf1a* is expressed in domains where PGCs are found and toward which they migrate in wild-type (Figure IIIB, C) (Doitsidou et al., 2002); PGCs migrate towards regions of ectopic Sdf1a expression (Doitsidou et al., 2002; Weidinger et al., 1999). Inhibition of the translation of Sdf1a, or its GPCR Cxcr4b, or mutations in their genes, result in severe PGC migration defects (Doitsidou et al., 2002; Knaut et al., 2003). Taken together, these findings indicate that this ligand and receptor pair of molecules provides a key directional cue for the PGCs as they migrate towards the future gonad. More recent studies showed that G *alpha* proteins of the Gi family are essential for directional migration but not for PGC motility (Dumstrei et al., 2004). Inhibition of phosphoinositide-3-kinase (PI3K) signaling in PGCs slows down their migration and leads to abnormal cell morphology as well as to the reduced stability of filopodia (Dumstrei et al., 2004). This data indicated that PI3K pathway is important for the motility of PGCs.

Besides the newly discovered roles of Sdf1a and its receptor Cxcr4b in the directed migration of zebrafish PGCs (Doitsidou et al., 2002; Knaut et al., 2003), the SDF1-CXCR4 interaction is also known to play roles in the chemotaxis of

chemosensory GPCRs (csGPCRs), serve as receptors for sensory signals of external origin, such as odorants, pheromones, and taste chemicals (Araneda et al., 2000; Buck and Axel, 1991; Mombaerts, 1999). Most other GPCRs respond to endogenous signals, such as peptides, lipids, neurotransmitters, and nucleotides (Howard et al., 2001). Hence they are referred to as endoGPCRs. GPCRs of this second-group are involved in numerous physiological processes, including the regulation of neuronal excitability, metabolism, reproduction, development, hormonal homeostasis, and behavior. In vertebrates, this superfamily contains 1000–2000 members (>1% of the genome) including >400 coding for endoGPCRs, which are well conserved in amino acid sequences between species (Bockaert and Pin, 1999). G protein-coupled receptors are also involved in many diseases and thus are major targets for pharmaceutical drugs (Vassilatis et al., 2003).

The roles of GPCRs in chemotaxis

The roles of GPCR in chemotaxis-based cell movements are well established by previous studies (Iijima et al., 2002; Parent and Devreotes, 1999). Chemotaxis is the phenomenon in which cells, bacteria, and other single-cell or multicellular organisms direct their movements according to concentration of certain chemicals in their environment. This is important for bacteria to find food (for example, glucose) by swimming towards the highest concentration of food molecules, or to flee from poisons (for example, phenol). In multicellular organisms, chemotaxis is critical to development as well as normal homeostasis

in adult organisms. In addition, it has been recognized that studying the mechanisms that regulating chemotaxis in various model organisms can be help us to understand cancer metastasis.

The molecular mechanisms of chemotaxis have been studied extensively in mammalian neutrophils and in the ameba *Dictyostelium discoideum*. G protein-coupled signaling has been shown to polarize neutrophils in response to chemoattractants. Upon exposure of cells to chemoattractant, the pleckstrin homology domain of the AKT protein kinase, tagged with the green fluorescent protein (PHAKT-GFP) is recruited selectively to membrane at the cell's leading edge (Servant et al., 2000). The more detailed understanding of establishment of cell polarity in chemotaxis was obtained in *Dictyostelium discoideum* (Iijima et al., 2002; Parent and Devreotes, 1999). In this model system, directional migration toward the chemoattractant is also controlled by asymmetric activation of a G protein-coupled receptor. Previous studies demonstrated mechanisms of navigation and signal amplification in chemotaxing *Dictyostelium* cells, in which phosphatidylinositol 3-kinase (PI3K) transiently translocates to the plasma membrane in response to chemoattractant stimulation and to the leading edge in chemotaxing cells. PTEN (phosphatase and tensin homolog), a negative regulator of PI3K pathways, exhibits a reciprocal pattern of localization. These findings revealed that differential subcellular localization and activation of PI3K and PTEN are required for proper chemotaxis (Funamoto et al., 2002; Iijima and Devreotes, 2002). The most important and interesting thing is that above discussed molecular cascades are presumably to be downstream of G-protein

coupled receptors.

GPCRs and vertebrate development

Whereas much is known about the roles of GPCRs in adult organisms, there is little existing knowledge about the involvement of GPCRs in vertebrate development. However, recent studies provide several potential links between GPCRs and coordinated cell migration during zebrafish embryogenesis. After gastrulation, the zebrafish *miles-apart* (*mil*) mutation specifically affects the migration of the heart precursors to the midline resulting in *cardia bifida*, such that two heart tubes form in mutants rather than one. Positional cloning showed that *mil* encodes a member of the lysosphingolipid GPCR family, revealing a new role for GPCRs in regulating cell migration during vertebrate development (Kupperman et al., 2000). Another example discussed previously involves the migration of PGCs. A pair of evolutionarily conserved molecules, the protein Sdf1a and its receptor Cxcr4b (a member of GPCRs), act to guide the migration of PGCs in zebrafish (Doitsidou et al., 2002). Also known as Odysseus, Cxcr4b was proven to be genetically required specifically in germ cells for their chemotaxis towards Sdf1a sources consistent with prediction of a receptor (Knaut et al., 2003). GPCRs have also been linked to chemotaxis in *Dictyostellium discoideum* and in the mammalian immune system (Devreotes and Janetopoulos, 2003). They have prominent roles in sensory organs and the central nervous system in adults. However, their roles in vertebrate gastrulation are not understood. Recent studies indicate that heterotrimeric G proteins, which

transduce signals downstream of GPCRs to regulate cell migration, are also essential for the gastrulation movements of epiboly, convergence, and extension in zebrafish (Lin et al., 2005). However, the identity of the corresponding GPCRs remains to be determined.

These interesting discoveries, along with similarities between the observations of the directed migration of mesodermal cells undergoing convergence movements (Jessen et al., 2002) and the established chemotaxis roles of GPCR in *Dictyosteliia* and immune cells, inspired us to investigate a subset of GPCRs from the hundreds of members in GPCR superfamily, and test their potential roles in vertebrate early development.

In this dissertation, I describe our discoveries regarding a pair of proteins, the Apelin ligand and its GPCR receptor Agtrl1b, in regulating cell movements during zebrafish early development. In Chapter II, I will describe that Apelin signaling is essential for heart formation, and regulates the migration of cardiac precursor cells during gastrulation. In Chapter III, I describe our results that reveal the role of Apelin signaling in regulating the migration of zebrafish PGCs.

CHAPTER II

APELIN AND ITS RECEPTOR CONTROL HEART FIELD FORMATION DURING ZEBRAFISH GASTRULATION

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Summary

The vertebrate heart arises during gastrulation as cardiac precursors converge from the lateral plate mesoderm territories toward the embryonic midline and extend rostrally to form bilateral heart fields. G-protein coupled receptors (GPCRs) mediate functions of the nervous and immune systems, however, their roles in gastrulation remain largely unexplored. Here we show that the zebrafish homologs of the Agtrl1b receptor and its ligand, Apelin, implicated in physiology and angiogenesis, control heart field formation. Zebrafish gastrulae express *agtrl1b* in the lateral plate mesoderm, while *apelin* expression is confined to the midline. Reduced or excess Agtrl1b or Apelin function caused deficiency of cardiac precursors and subsequently heart. In Apelin deficient gastrulae, the cardiac precursors converged inefficiently to the heart fields and showed ectopic distribution, whereas cardiac precursors overexpressing Apelin exhibited abnormal morphology and rostral migration. Our results implicate GPCR signaling in movements of discrete cell populations that establish organ rudiments during vertebrate gastrulation.

Introduction

The vertebrate body plan, germ layers and organ rudiments are established during gastrulation via concurrent inductive and morphogenetic events. Internalization generates the three germ layers when endodermal and

mesodermal precursors move beneath the prospective ectodermal layer. The process of epiboly spreads and thins the germ layers. Convergence movements narrow the germ layers mediolaterally while extension movements elongate them anteroposteriorly. Several signaling pathways have been implicated in directing these large-scale gastrulation movements in vertebrates, including Stat3, non-canonical Wnt and FGF signaling (Keller, 2005; Leptin, 2005; Solnica-Krezel, 2005). G-protein coupled receptors (GPCRs) have been linked to chemotaxis in *Dictyostellium discoideum* and in the mammalian immune system (Devreotes and Janetopoulos, 2003). They have prominent roles in sensory organs and central nervous system in adults. However, their roles in vertebrate gastrulation are not understood. In fact little is known about the developmental expression and/or function of any of the close to 400 endoGPCRs encoded in the human genome (Vassilatis et al., 2003). Recent studies indicate that heterotrimeric G proteins, which transduce signals downstream of GPCRs to regulate cell migration, are also essential for the gastrulation movements of epiboly, convergence and extension in zebrafish (Lin et al., 2005). However, the identity of the corresponding GPCRs remains to be determined.

Here we implicate a chemokine Apelin and its receptor Agltr1b in convergence and extension gastrulation movements of cardiac precursors in zebrafish. Agtrl1 (also named APJ), initially identified as an ‘‘orphan’’ receptor, was shown to be activated by a 36-amino-acid peptide from bovine stomach homogenates (Tatemoto et al., 1998). Subsequently, a 77-amino-acid prepropeptide of a novel ligand, called Apelin for Agtrl1 endogenous ligand, was

identified in human and bovine tissues. Peptides of varying size comprising C-terminal fragments of the Apelin prepropeptide can activate the receptor (Habata et al., 1999; Kawamata et al., 2001; Lee et al., 2000). Several studies have reported cardiovascular actions of Apelin/Agtrl1 in humans, including regulation of blood pressure *in vivo* (Tatemoto et al., 2001), and exertion of positive inotropic effects in the heart (Berry et al., 2004; Szokodi et al., 2002). Reports of declining Apelin/Agtrl1 levels in patients with chronic heart failure imply this signaling system may have cardioprotective properties (Chen et al., 2003). More recently, *apelin* has been shown to be required for normal vascular development in frog embryos (Inui et al., 2006b).

The heart anlage arises during gastrulation when prospective cardiac precursors, specified in the lateral plate mesoderm territories, converge toward the embryonic midline and extend rostrally to form bilateral heart fields at late gastrulation (Keegan et al., 2004). The two heart fields fuse during segmentation into a single heart tube (Auman and Yelon, 2004; Moorman and Christoffels, 2003; Yelon and Stainier, 1999). In zebrafish, lysosphingolipid signaling via the Miles apart GPCR is essential for movements and fusion of the bilateral heart primordia during segmentation (Kupperman et al., 2000). Whether similar pathways that regulate movements of defined cell populations also operate during vertebrate gastrulation is an open question.

We provide several lines of evidence that Apelin and its Agtrl1b receptor regulate heart precursor cell movements during zebrafish gastrulation. Whereas *agtrl1b* is expressed in the lateral plate mesoderm, *apelin* expression is confined

to the midline, where signals regulating convergence and extension movements are thought to reside (Solnica-Krezel, 2005). Reduction or excess Agtrl1b or Apelin expression impaired cardiac precursor and heart formation, whereas other embryonic tissues were only mildly affected. In gastrulae overexpressing or deficient in Apelin, the cardiac precursors moved inefficiently to the heart fields and exhibited abnormal morphology and protrusive activity.

Our work uncovers a novel role of Apelin/Agtrl1b signaling in mediating heart field formation during zebrafish gastrulation. These findings implicate GPCR signaling in cell movements that establish organ rudiments during gastrulation and suggest that vertebrate gastrulation employs pathways that govern movements of all gastrula cells or entire germ layers, as well as pathways that regulate movements of discrete cell populations.

Results

Complementary expression of Agtrl1b receptor and its ligand Apelin in zebrafish gastrulae

In our efforts to identify GPCRs regulating vertebrate gastrulation we isolated zebrafish homologs of Angiotensin II receptor-like 1 (Agtrl1), *agtrl1b* and *agtrl1a* and its putative ligand Apelin (*apln*). *agtrl1a* is not described here, but its cloning and expression pattern has been recently reported (Tucker et al., 2007). Agtrlb and Apelin have been previously identified in human, mouse and *Xenopus laevis* (Figure S1) (Devic et al., 1996; Devic et al., 1999; O'Dowd et al., 1993).

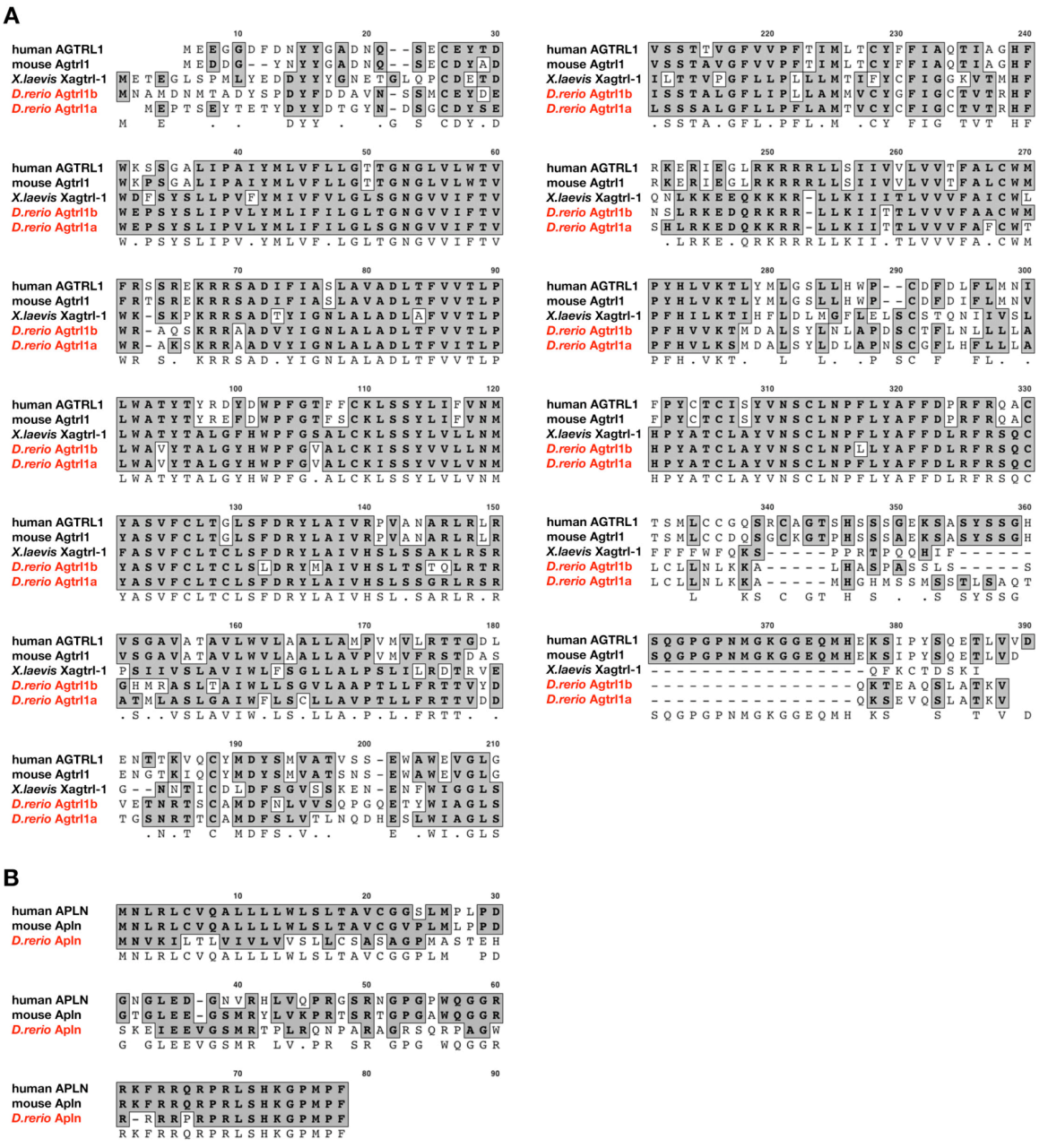
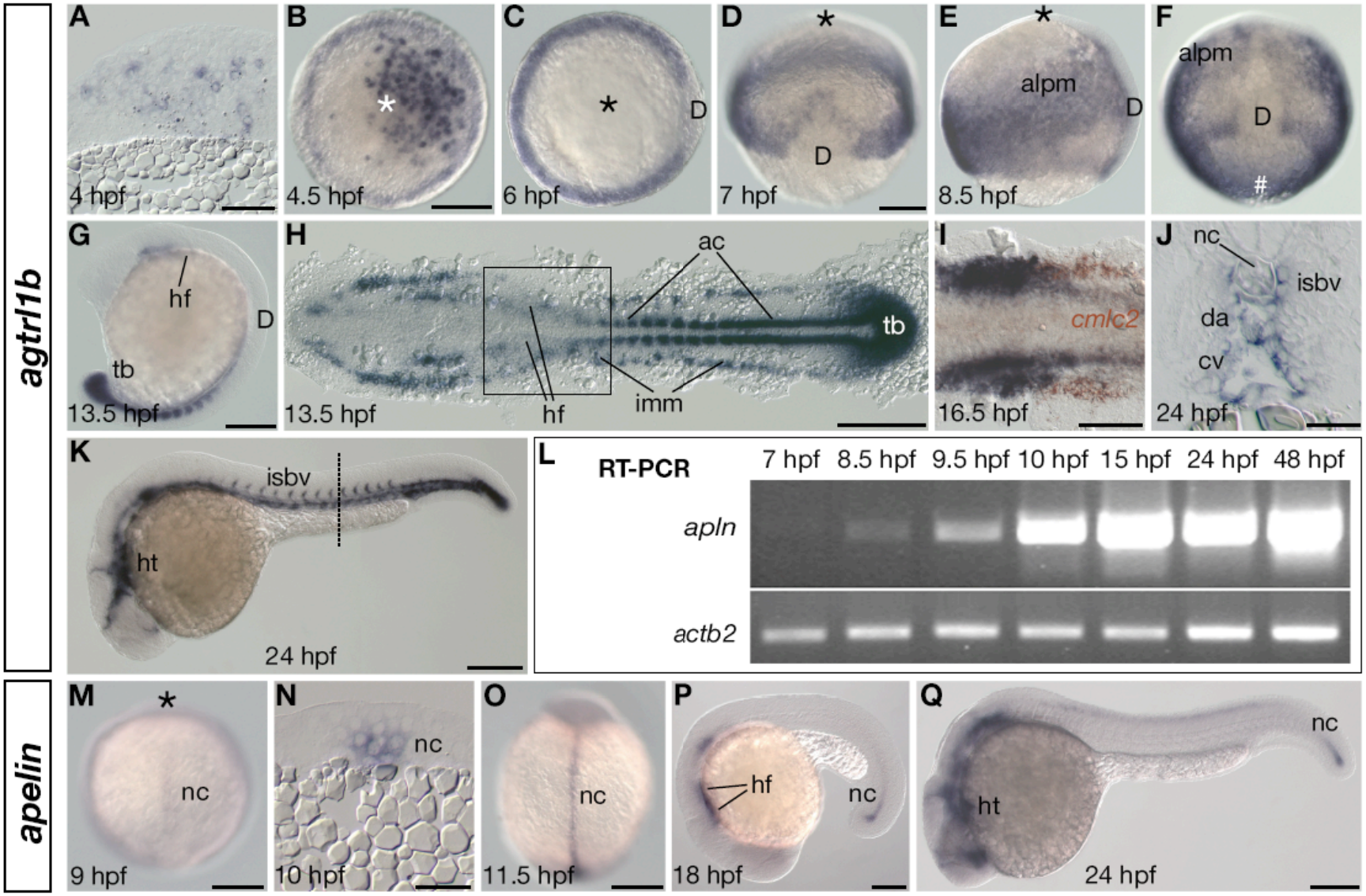


Figure S1. Sequence alignment of Agtrl1b (A) and Apelin proteins (B). Human (AGTRL1, APLN), mouse (Agtrl1, Apln), *Xenopus laevis* (Xagtrl-1/Xmsr-1) and zebrafish Agtrl1b/Agtrl1a and Apln. Identical sequences are boxed in grey.

Figure 1. Spatiotemporal expression of *agtrl1b* and *apln* during zebrafish embryogenesis.
(A-K) *agtrl1b* expression profile. (L-Q) *apln* expression profile. (A) *agtrl1b* expression in deep cells at 4 hpf. (B) At 4.5 hpf *agtrl1b* expression appears at blastoderm margin. (C) At onset of gastrulation, *agtrl1b* expression is only maintained in the blastoderm margin. (D-F) *agtrl1b* expression during gastrulation in the anterior lateral plate mesoderm (alpm), and posterior and adaxial mesoderm. (G, H) New *agtrl1b* expression domains in the developing brain, adaxial cells (ac), intermediate mesoderm (imm) and heart field (hf) at early segmentation stages (8 somites); tailbud (tb); (G) lateral view; (H) flat mount, anterior to the left. (I) Boxed region in (H), by midsegmentation (14 somites) *agtrl1b* expression in cardiac precursors overlaps with *cmlc2* (red). (J, K) New *agtrl1b* expression in dorsal aorta (da), caudal vein (cv) and intersomitic blood vessels (isbv) at 24 hpf. (J) Cross section through trunk region marked by dashed line in (K); notochord, (nc).
(l) RT-PCR of *apln* transcript at 7-48 hpf; *β-actin* (*actb2*) was used as a loading control. (M-O) *apln* expression in the axial mesoderm and its derivative, notochord. (N) Cross section through notochord. (P, Q) *apln* expression in posterior notochord, prospective heart (ht) and within the head at 24 hpf. Animal pole (☆,★). Vegetal pole (#). Dorsal (D). Scale bar represents 100 μm (A, I, J, N) and 200 μm (B, D, G, H, K, M, O, P, Q).


agtrl1b
A
4 hpf
B
4.5 hpf
C
6 hpf
D
7 hpf
E
8.5 hpf
alpm
F
alpm
D
#
G
hf
tb
13.5 hpf
H
ac
tb
hf
imm
13.5 hpf
I
cmlc2
16.5 hpf
J
nc
isbv
da
cv
24 hpf
K
isbv
ht
24 hpf
L
RT-PCR
7 hpf 8.5 hpf 9.5 hpf 10 hpf 15 hpf 24 hpf 48 hpf
apln
actb2
apelin
M
nc
9 hpf
N
nc
10 hpf
O
nc
11.5 hpf
P
hf
nc
18 hpf
Q
nc
ht
24 hpf

Our analysis of the spatiotemporal *agtrl1b* and *apln* expression suggested involvement of these molecules in gastrulation and heart development. By whole mount *in situ* hybridization, *agtrl1b* expression was first detected after the onset of zygotic transcription in randomly positioned cells in the blastoderm (Figure 1A). By 4.5 hours post fertilization (hpf), mesendodermal precursors at the blastoderm margin started to express *agtrl1b* (Figure 1B). At early gastrulation marginal *agtrl1b* expression was maintained ventrolaterally, while expression in dorsal and in random deep cells declined (Figures 1C, D). During gastrulation and segmentation, *agtrl1b* expression was maintained in adaxial, intermediate, and lateral plate mesoderm (LPM), including the anterior LPM where the heart precursors reside (Figures 1E, F) (Keegan et al., 2004). During late segmentation, several *agtrl1b* expression domains were detected, including in the forming heart, as revealed by co-expression with the heart marker *cardiac myosin light chain 2* (*cmlc2*) (Figures 1G - I). By 24 hpf, *agtrl1b* expression was detected in dorsal aorta, caudal vein, and intersomitic blood vessels (Figures 1J, K). Expression in blood vessels declined by 1.5 days post fertilization (dpf), and was undetectable at 2 dpf (not shown).

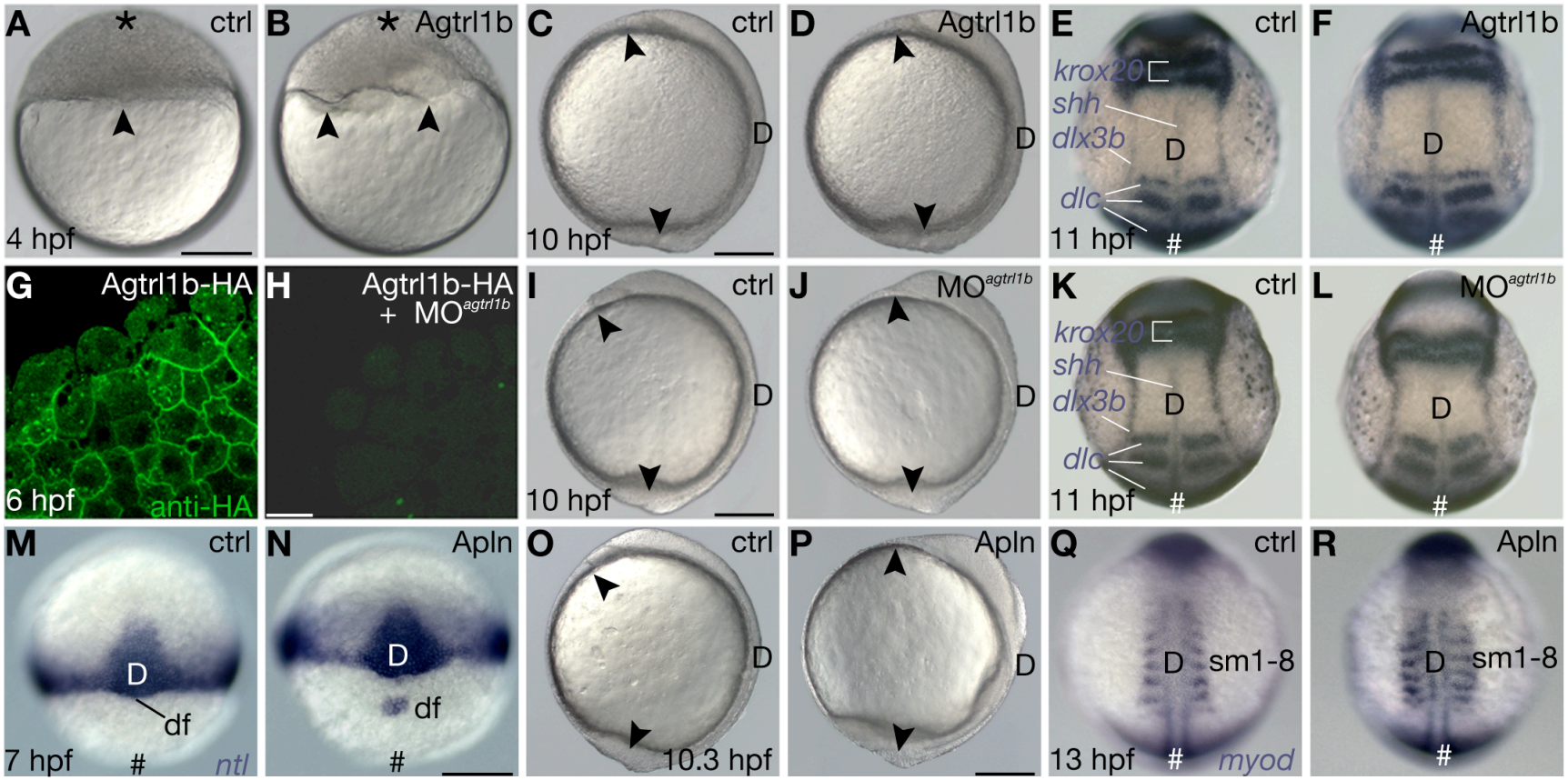
Transcripts of the Agtrl1b ligand, Apelin, were first detected at midgastrulation by RT-PCR (Figure 1L). Interestingly, whole mount *in situ* hybridization revealed that *apln* expression complemented that of *agtrl1b* during gastrulation: *apln* transcripts were detected exclusively in the axial mesoderm and its later derivative, the notochord (Figures 1M-O). At late segmentation *apln*

expression was maintained posteriorly in the forming notochord (Figure 1P), and new expression was identified in the presumptive heart, coinciding with expression of its receptor (Figures 1P, I, K). By 24 hpf, discrete *apln* expression domains appeared in the head (Figure 1Q).

Excess and deficit of Agtrl1b impair gastrulation and heart formation

To investigate the role of Apelin and its receptor during zebrafish development we misexpressed the Agtrl1b receptor by microinjecting synthetic RNA into 1-cell stage embryos. The earliest effects were detected at 4 hpf, when the interface between the blastoderm and the yolk cell showed strong distortions (Figures 2A, B). As *apelin* is not expressed until 7 hpf and constitutive activity in a ligand independent manner was reported for G-protein-coupled receptors (Smit et al., 2006), we attribute these effects to excess receptor activity. Subsequently the progress of epiboly and other gastrulation movements were variably compromised (not shown). Nevertheless, by the end of the gastrula period, all injected embryos manifested normal AP axis extension, although 85% exhibited mediolateral broadening of neuroectodermal and mesodermal tissues compared to uninjected siblings (n=265, 400 pg; Figures 2C-F) and by 24 hpf injected embryos were of normal length or slightly shorter (not shown). Analyses of tissue-specific markers revealed that embryonic patterning and tissue specification were not compromised by the injection of *agtrl1b* RNA, except for a small reduction in myocardial marker expression (n=234, 400 pg; Figures 2E, F;

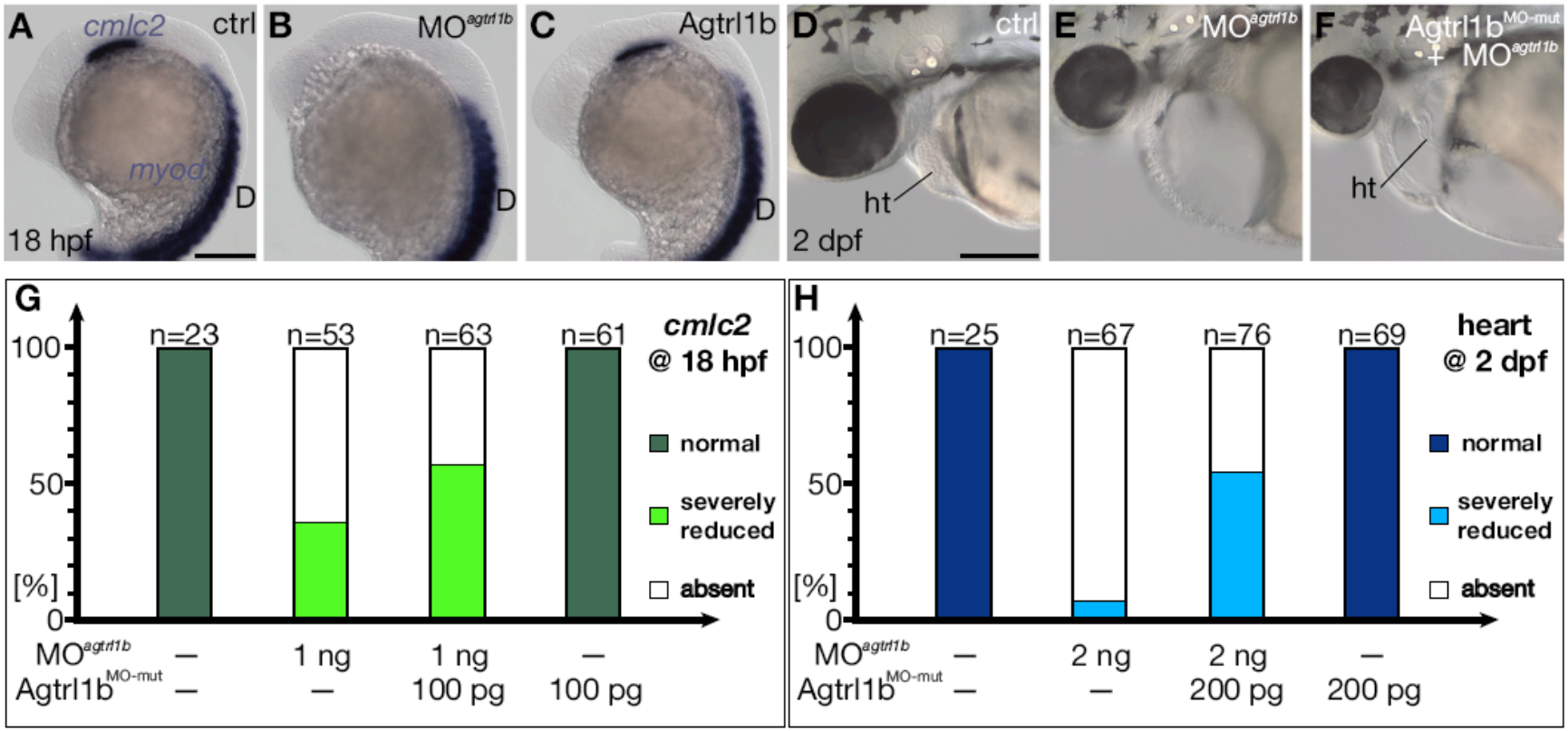
Figure 2. *agtrl1b* and *apelin* can influence gastrulation movements. (A-F) *agtrl1b* gain-of-function and (G-L) loss-of-function. (A, B) Agtrl1b misexpression (B) causes distortion at the interface between blastoderm and yolk cell at early epiboly, in contrast to smooth yolk surface indicated by an arrowhead in uninjected control embryo (A). (C, D) By the end of gastrulation embryos overexpressing Agtrl1b (D) show normal anteroposterior extension; arrowheads indicate anterior and posterior limits of the nascent embryonic axis. (E, F) Tissue specification and patterning as revealed by *krox20* (hindbrain rhombomeres 3 and 5), *deltaC* (*dlc*, newly formed somites), *dlx3b* (neural-nonneural ectoderm boundary) and *shh* (notochord) expression. These markers reveal mediolateral expansion of tissues, consistent with reduced convergence gastrulation movements in Agrl1b misexpressing embryos (F). (G, H) Evaluation of $MO^{agtrl1b}$ effectiveness. Confocal microscopy image of cell membrane localized HA-tagged Agtrl1b in blastulae injected with its synthetic RNA. (H) Co-injection of $MO^{agtrl1b}$ suppresses ectopic HA-tagged Agtrl1b expression. (I-L) Embryos injected with $MO^{agtrl1b}$ manifest a mild reduction of anteroposterior embryonic axis and a normal mediolateral expansion at the end of gastrulation. Tissue specification and patterning, marked by *krox20*, *dlc*, *dlx3b* and *shh* expression, are not affected (K, L). (M-R) Effect of Apelin misexpression on early gastrulation. (M, O, Q) Uninjected control embryos (ctrl). (N, P, R) Embryos injected with 10 pg *apln* RNA (Apln). (M, N) Dorsal views, 7 hpf, animal pole to the top. *no tail (ntl)* expression in mesoderm at the blastoderm margin/blastopore marks the progress of epibolic movements towards the vegetal pole (#); dorsal forerunner cells (df) are well separated in *apln* RNA-injected embryos, revealing impaired epiboly of the blastoderm margin (N). (O, P) Lateral view, 1-somite stage, 10.3 hpf. The anteroposterior axis marked with arrowheads is reduced in *apln* RNA injected embryos. (Q, R) Dorsal view, 8-somite stage, animal pole (★) to the top. *myod* marks formed somites, which are expanded mediolaterally in *apln* RNA-injected embryos (R). Dorsal (D). Scale bar represents 5 μm (H), and 200 μm (A, C, I, N, P).


A
ctrl
4 hpf
B
Agtrl1b
C
ctrl
10 hpf
D
Agtrl1b
E
ctrl
krox20
shh
dlx3b
dlc
D
11 hpf
#
F
Agtrl1b
G
Agtrl1b-HA
6 hpf
anti-HA
H
Agtrl1b-HA
+ MO agtrl1b
I
ctrl
10 hpf
J
MO agtrl1b
K
ctrl
L
MO agtrl1b
M
ctrl
df
7 hpf
ntl
N
Apln
O
ctrl
10.3 hpf
P
Apln
Q
ctrl
sm1-8
13 hpf
myod
R
Apln

3A, C; and not shown). These results suggest that correct expression level and spatial distribution of Agtrl1b are crucial for normal gastrulation movements.

We assessed the requirement for Agtrl1b during gastrulation using antisense morpholino oligonucleotides ($MO^{agtrl1b}$) designed to interfere with its translation (Nasevicius and Ekker, 2000). Whereas all embryos injected with synthetic RNA encoding carboxyl terminus HA-tagged Agtrl1b alone exhibited membrane-bound receptor (n=5), Agtrl1b-HA expression was downregulated in embryos co-injected with $MO^{agtrl1b}$ (n=10; Figures 2G, H), supporting the effectiveness of $MO^{agtrl1b}$ in inhibiting Agtrl1b translation. Embryos injected with 1 or 2 ng of $MO^{agtrl1b}$ showed at late gastrulation an almost normal mediolateral axis with a dose-dependent reduction of AP axis (Figures 2I, J and not shown), but only minor changes in general tissue specification and patterning (Figures 2K, L). Strikingly, at segmentation stages, the expression of *cmlc2* in cardiac precursors was strongly reduced or missing (Figures 3A, B, G). At 2 dpf a functional heart was not detected in the vast majority of *agtrl1b* morphant embryos while the body length appeared normal (Figures 3D, E, H). To test the specificity of this phenotype, we co-injected sequence modified *agtrl1b* RNA ($agtrl1b^{MO\text{-}mut}$) that should not bind $MO^{agtrl1b}$. Indeed, 54% of the embryos co-injected with $MO^{agtrl1b}$ and the MO resistant RNA showed partial suppression of the $MO^{agtrl1b}$ – dependent phenotype, exhibiting a small beating heart (Figure 3F). By contrast, 93% percent of the embryos injected with $MO^{agtrl1b}$ alone failed to form a functional heart (Figure 3H). Consistently, 57% of these embryos co-injected with $MO^{agtrl1b}$ and rescuing mRNA exhibited faint *cmlc2* staining,

Figure 3. *agtrl1b* is essential for heart formation.
(A-H) *agtrl1b* loss-of-function experiments. Downregulation of Agtrl1b expression causes deficiency of cardiac precursors marked by *cmlc2* (B), compared to uninjected control embryo (A), without affecting somitic *myod* expression. (C) Ectopic Agtrl1b activity mildly impairs cardiac precursor development. (D, E) Functional heart (ht) does not form in *agtrl1b* morphant embryos, but residual heart is observed in embryos co-injected with $MO^{agtrl1b}$ and 200 or 100 pg of MO-resistant synthetic *agtrl1b* RNA (F). (G-H) Evaluation of $MO^{agtrl1b}$ specificity scored by *cmlc2* expression at midsegmentation (G) or morphology at 2 dpf (F, H). Dorsal (D). Scale bar represents 100 μm (D-F) and 200 μm (A-C).


A
cmlc2
ctrl
myod
D
18 hpf
B
MO agtrl1b
D
C
Agtrl1b
D
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2 dpf
E
MO agtrl1b
F
Agtrl1b MO-mut
+ MO agtrl1b
ht
G
100
50
[%]
0
n=23
n=53
n=63
n=61
cmlc2
@ 18 hpf
normal
severely reduced
absent
MO agtrl1b — 1 ng 1 ng —
Agtrl1b MO-mut — — 100 pg 100 pg
H
100
50
[%]
0
n=25
n=67
n=76
n=69
heart
@ 2 dpf
normal
severely reduced
absent
MO agtrl1b — 2 ng 2 ng —
Agtrl1b MO-mut — — 200 pg 200 pg

compared to 36% of the embryos that were injected with MO^{agtrl1b} alone (Figure 3G). The incomplete suppression of this phenotype may be explained in part by the observation that both expression of high dose (400 pg, Figure 3C) and depletion of Agtrl1b caused cardiac deficiency. Taken together, these experiments revealed an essential role of Agtrl1b in heart formation.

Excess and deficit of Apelin impair gastrulation and heart formation

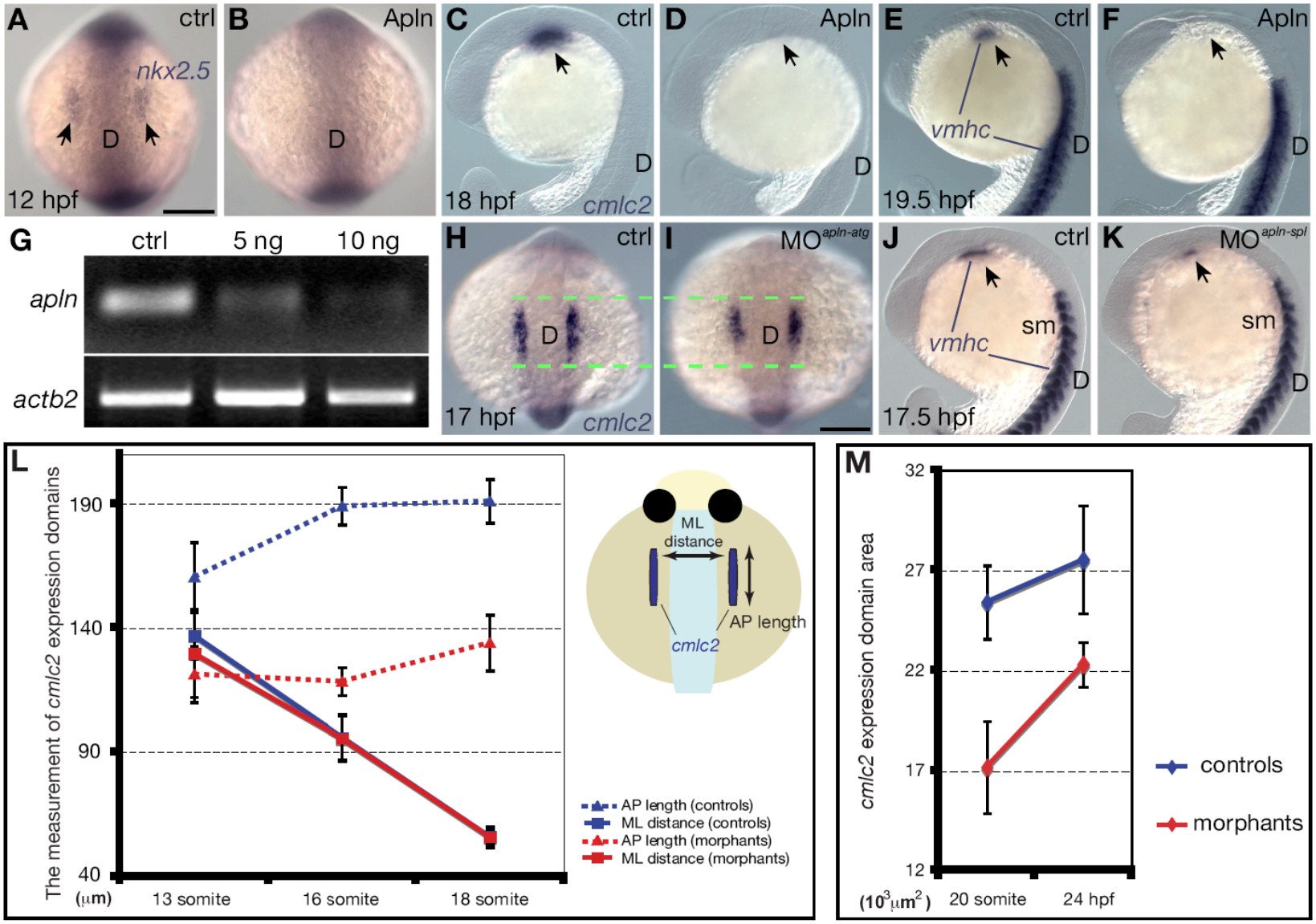
We next tested the involvement of the ligand, Apelin, in gastrulation and heart development. Excess Apelin impaired epiboly, a process that involves coordinated movements of the blastodermal layers towards the vegetal pole to enclose the yolk cell. In 73% (n=122) of the embryos injected with 10 pg of synthetic RNA encoding the Apelin prepropeptide (Lee et al., 2000), movement of the blastoderm towards the vegetal pole was delayed (Figures 2M, N). Moreover, dorsal forerunner cells, a small cell cluster that originates from the dorsal blastoderm margin and moves vegetally in front of the blastoderm, were well separated from the delayed blastoderm (Figures 2M, N). Hence, excess Apelin interferes with epibolic movements of most deep cells during gastrulation. During early segmentation 91% (n=221) of the embryos misexpressing Apelin showed reduction of the AP axis (Figures 2O, P) and mediolaterally expanded somites (Figures 2Q, R), suggesting that excess Apelin also impairs convergence and extension movements. However, tissue specification and patterning during gastrulation were not significantly altered (Figures 2Q, R and

not shown). At late segmentation, embryos misexpressing Apelin displayed a complete lack of the heart markers *nkx2.5* (Figures 4A, B), *cmlc2* (Figures 4C, D) and *ventricle myosin heavy chain* (*vmhc*) (Figures 4E, F) despite having relatively normal morphology and expression of *vmhc* in the somites (Figures 4E, F). Furthermore, these embryos did not exhibit a beating heart at 2 dpf (not shown). At 30 hpf embryos misexpressing Apelin also displayed reduced expression of several anterior LPM markers including *hand2*, *gata5*, *tbx1* as well as *nkx2.3* in pharyngeal pouch mesenchyme and of *tbx5* in pectoral fin, indicating that other anterior LPM derivatives were affected (Figure S2 and not shown).

To determine which developmental processes require Apelin, we designed MOs to interfere with either protein translation ($MO^{apln\text{-}atg}$) or RNA splicing ($MO^{apln\text{-}spl}$). Binding of $MO^{apln\text{-}spl}$ should cause an insertion of intron1-derived sequences predicted to create a premature stop codon following the first 27 amino acids of Apelin, which would therefore lack the carboxyl terminal sequence essential for receptor binding and activation (Hosoya et al., 2000; Kawamata et al., 2001; Lee et al., 2000). Injection of 10 ng of $MO^{apln\text{-}spl}$ was sufficient to suppress normal splicing of endogenous *apln* RNA as revealed by RT-PCR at 12, 24 and 32 hpf (Figure 4G and not shown). Moreover, $MO^{apln\text{-}atg}$ suppressed the epiboly defects caused by injection of *apln* RNA, but did not affect the phenotype caused by a mutated form of the *apln* RNA that lacked sequences for $MO^{apln\text{-}atg}$ binding (not shown).

Embryos injected with either 1 ng of $MO^{apln\text{-}atg}$ or 10 ng of $MO^{apln\text{-}spl}$ exhibited the same phenotype, characterized by reduced expression of

Figure 4. Excess and deficiency of Apelin impair heart formation.
(A-F) *apelin* gain-of-function and (G-M) loss-of-function experiments. (A, C, E, H, J) control embryos (ctrl). (B, D, F) Embryos injected with 10 pg *apln* RNA (Apln). (I) Embryos injected with 1 ng MO$^{apln\text{-}atg}$. (K) Embryos injected with 10 ng MO$^{apln\text{-}spl}$. (A, B) Dorsal view, *nkx2.5* expression in cardiac precursors (arrows) is not detected in Apelin misexpressing embryos at midsegmentation. (C-F) Expression of *cmlc2* (C, arrow) and *vmhc* (E, arrow) in heart primordia of late segmentation control embryos is not detected in Apelin misexpressing siblings (D, F, arrows). (E, F) Normal *vmhc* expression in somites (sm) of control and Apelin misexpressing siblings. (G) RT-PCR amplified *apln* fragment from uninjected embryos (lane ctrl) at 32 hpf, and embryos injected with *apln* MO$^{apln\text{-spl}}$ at 5 ng and 10 ng doses. β-*actin* 2 (*actb2*), was used as a loading control. (H-K) Reduced expression of cardiac markers *cmlc2* and *vmhc* (arrows), but not somitic *vmhc* expression (sm) in embryos depleted of Apelin by MO$^{apln\text{-atg}}$ or MO$^{apln\text{-spl}}$. Dorsal (D). Scale bar represents 200 μm (A, I).
Measurement of the anteroposterior dimension and the mediolateral distance between the two *cmlc2* expression domains (L) in uninjected control embryos and *apln*^{spl} morphants at three developmental stages before the fusion of cardiac primordia (13, 16 and 18 somite). AP: anteroposterior, ML: mediolateral. (M) After bilateral heart primordia fused together, the area of *cmlc2* expression domain was measured at 20 somite and 24 hpf in control and *apln*^{spl} morphant embryos. Each point represents the average measurement of 10 embryos. Error bars depict standard deviation.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The measurement of cmlc2 expression domains
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140
90
40
(μm)
13 somite
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18 somite
ML distance
AP length
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AP length (controls)
ML distance (controls)
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ML distance (morphants)
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22
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(10³μm²)
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24 hpf
controls
morphants

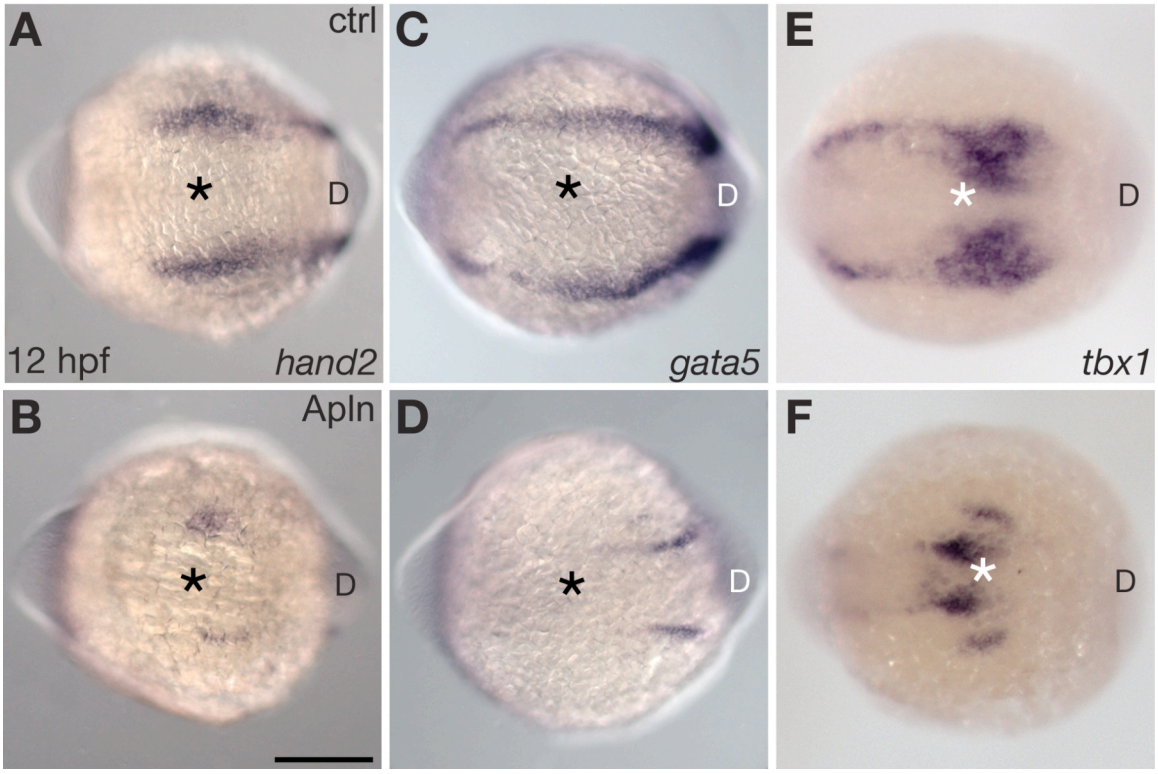


Figure S2. Excess Apelin inhibits heart cell fate in the anterior LPM. *hand2 (*A, B), *gata5* (C, D) or *tbx1* (E, F) are expressed in continuous bilateral stripes in the anterior LPM at 12hpf, coinciding with cardiac precursors in control embryos (A, C, E). These genes exhibited reduced and patchy expression in the anterior LPM when Apelin was ubiquitously expressed (B, D, F). Animal pole (★). Dorsal (D). Scale bar represents 200 μm (B).

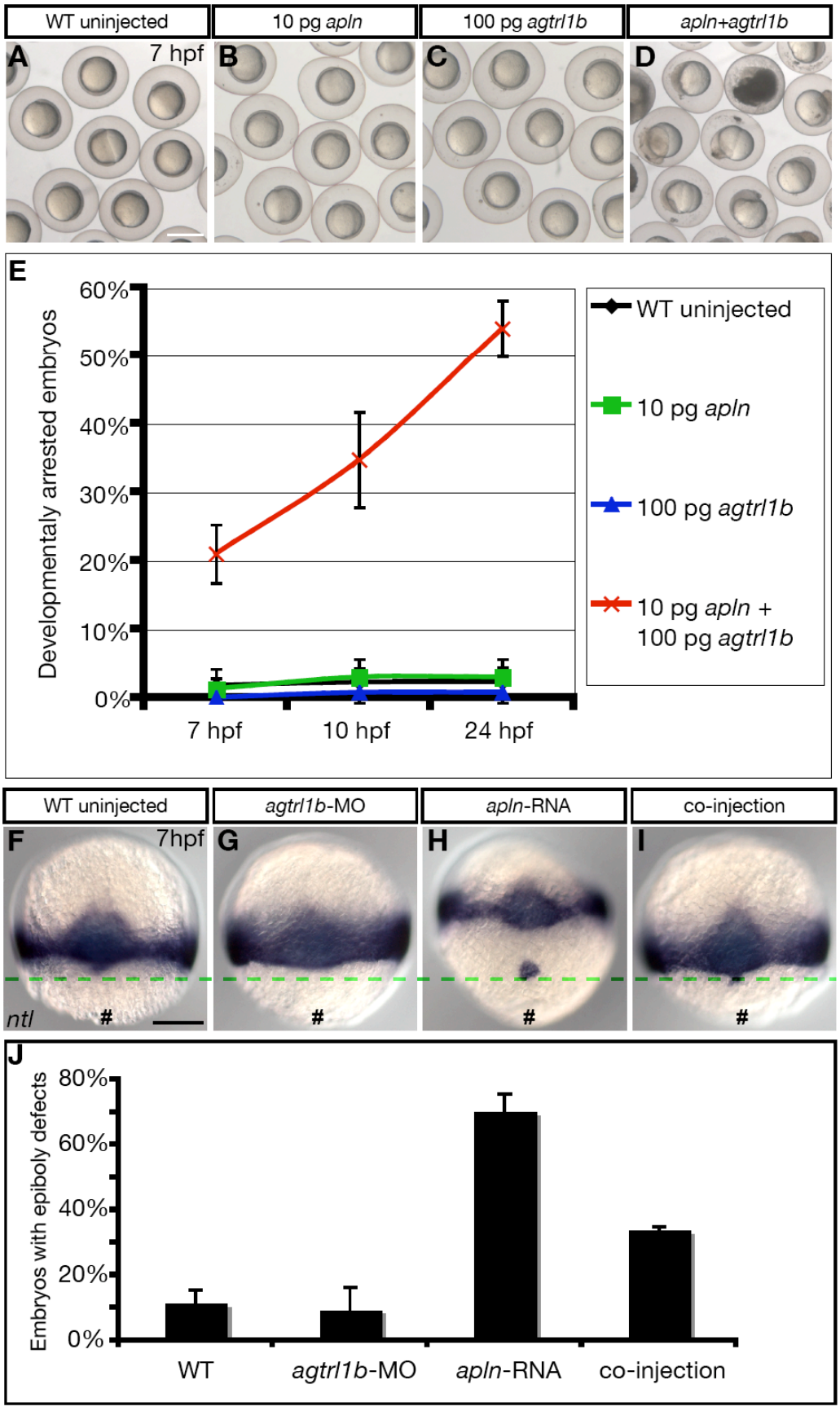
myocardial markers including *cmlc2* (84%, n=63, and 81%, n=62, respectively) and *vmhc* (83%, n=58, and 96%, n=45, respectively) (Figures 4H-M and not shown), despite normal *vmhc* expression in the somites (Figure 4J, K). However, the MO$^{apln\text{-}spl}$ embryos showed neither a delay in the fusion of bilateral heart primordia (Figures 4L, M) nor cardia bifida at 1 dpf (not shown).

Apelin signals through Agtrl1b during gastrulation

Since interference with Apelin or Agtr1b impaired cardiac precursor formation, we asked whether Apelin signals via the Agtrl1b receptor in zebrafish embryos, as reported for its mammalian homologs (Lee et al., 2000; Tatemoto et al., 1998). We reasoned that ligand and its receptor should have synergistic effects on development in co-injection experiments. Accordingly, embryos injected separately with low doses of synthetic RNAs encoding Agtrl1b or Apelin progressed through gastrulation, whereas embryos co-injected with the same doses of both RNAs underwent developmental arrest by late blastula stages and most died by 1 dpf (Figures 5A-E). We also expected that Agtrl1b function should be required for excess Apelin to impair gastrulation movements. Accordingly, injections of MO^{agtrl1b} significantly suppressed the epiboly delay caused by Apelin misexpression (Figures 5F-J). Together, these results provide strong support for the notion that during zebrafish gastrulation Apelin functions upstream of Agtrl1b receptor, likely as its specific ligand.

Excess or deficit of Apelin impair convergence and extension movements of anterior LPM and heart precursors

Figure 5. Functional interaction of Apelin and Agtrl1b during zebrafish gastrulation.
(A-E) Co-injection of synthetic *apln* and *agtrl1b* RNAs leads to developmental arrest in a synergistic fashion. Injection of low doses of synthetic *apln* and *agtrl1b* RNAs alone rarely caused developmental arrest (B, C), as observed for uninjected wild-type embryos (A). Embryos co-injected with the same doses of synthetic *apln* and *agtrl1b* RNAs underwent developmental arrest by late blastula stages (D). Graph (E) shows the fraction of developmentally arrested embryos at different times after fertilization in the above experiments. Data is from three separate experiments: uninjected wild-type embryos (n=213), 10 pg *apln* RNA injected (n=199), 100 pg *agtrl1b* RNA injected (n=189), and embryos co-injected with 100 pg *agtrl1b* RNA and 10 pg *apln* RNA (n=194). Error bars depict standard deviation.
(F-J) Injection of *agtrl1b* MO suppresses epiboly defects caused by ectopic Apelin expression. Top panel, *no tail* (*ntl*) expression marks mesendodermal margin and dorsal forerunner cells in uninjected control embryos (F), 2 ng $MO^{agtrl1b}$ injected (G), 10 pg *apln* RNA injected (H) and 2 ng $MO^{agtrl1b}$ and 10 pg *apln* RNA co-injected (I) embryos. Dorsal forerunner cells are tightly associated with the blastoderm margin in uninjected wild type (F) and $MO^{agtrl1b}$ injected (G) embryos, while embryos overexpressing Apelin (H) exhibit defective epiboly demonstrated by the separation of dorsal forerunner cells from the blastoderm margin and delayed movement of the margin towards the vegetal pole. Embryo co-injected with *apln* RNA and $MO^{agtrl1b}$ shows a significant suppression of the epiboly defect (I). Graph (J) depicts the percentage of embryos with epiboly defects assessed by *ntl* staining. Data is from three experiments: uninjected wild-type embryos (n=83), 2 ng $MO^{agtrl1b}$ injected (n=59), 10 pg *apln* RNA injected (n=65), and the embryos co-injected with 2ng $MO^{agtrl1b}$ with 10 pg *apln* RNA (n=69). Error bars depict standard deviation. Vegetal pole (#). Scale bar represents 200 μm (F) and 600 μm (A).


WT uninjected
10 pg apln
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apln+agtrl1b
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Developmentaly arrested embryos
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20%
10%
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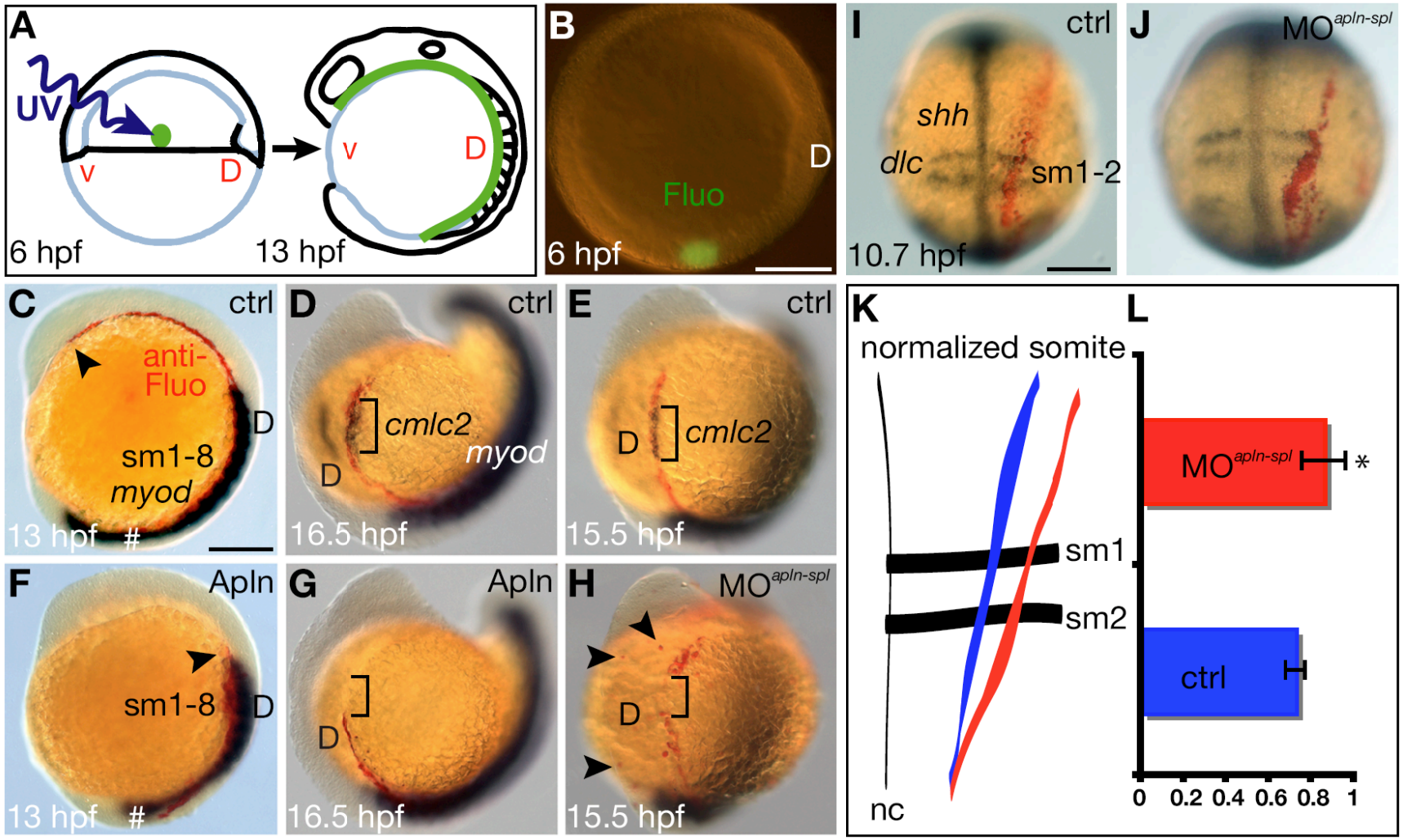
7 hpf
10 hpf
24 hpf
WT uninjected
10 pg apln
100 pg agtrl1b
10 pg apln +
100 pg agtrl1b
WT uninjected
agtrl1b-MO
apln-RNA
co-injection
F
7hpf
G
H
I
ntl
#
Embryos with epiboly defects
80%
60%
40%
20%
0%
WT
agtrl1b-MO
apln-RNA
co-injection

The deficiency of cardiac gene expression in embryos with reduced or excess Apelin could result from loss of a localized source of Apelin signal to guide the cardiac precursors to the correct location. Indeed, guided cell migration can be compromised by both a deficit and an excess of cues (Doitsidou et al., 2002; Duchek and Rorth, 2001; Solnica-Krezel, 2005). Alternatively, loss of heart precursors could reflect defective cell fate specification or survival. To address these possibilities we performed cell tracing experiments (Figures 6A-J; S3A-D) (Sepich and Solnica-Krezel, 2005). Mesendodermal cell groups, positioned at the lateral blastoderm margin that give rise to the cardiac precursors (Keegan et al., 2004), were labeled by photoactivation of caged fluorescein at the beginning of gastrulation (Figures 6A, B). The embryos were fixed during segmentation and the labeled cells were visualized with anti-fluorescein antibody, while somitic and heart precursors were detected using antisense RNA probes (*myod, cmlc2*, Figures 6C, D, F, G; *deltaC*, Figures S3A-D). In control embryos as previously described for convergence and extension movements of the lateral mesoderm (Myers et al., 2002), the labeled cell populations converged towards the midline and extended from the tail, through the heart field and to the most anterior part of the embryo, forming a fine stripe (n=37, Figures 6C, D; S3A, B). Measurements revealed that the labeled cell array extended 886.5 ± 54.3 μm from the 8^{th} somite to the anterior end (n=19; Figures S3A). In contrast, in embryos injected with 10 pg of synthetic *apln* RNA the labeled cell population converged to the dorsal midline and reached comparable position within the tail, but failed to extend anteriorly and to express *cmlc2* (n=44, Figures 6F, G; S3C, D). The length of

Figure 6. Abnormal convergence and extension movements of lateral mesoderm cells in embryos with excess and deficit of Apelin.
(A) The method for labeling cell populations by photo-activation of caged FITC-dextran is illustrated. A prospective mesodermal cell population is labeled at the lateral blastoderm margin at the start of gastrulation (6 hpf). The labeled cell group is followed through gastrulation as it elongates and converges toward the dorsal midline and extends anteroposteriorly. (B) Image of an embryo with the animal pole to the top and dorsal to the right in which a group of labeled cells positioned 90° from the dorsal midline is visualized under the fluorescent channel and Nomarski optics. (C-H, I and J) Tracing fates and movements of lateral mesoderm cells in uninjected control embryos (C, D, E, I), embryos with excess (F, G) and deficit of Apelin (H, J). (C, F) Lateral views, (I, J) dorsal views and (D, G, E, H) dorso-anterior views at 2 somite (I, J), 8 somite (C, F), 13 somite (E, H) and 15 somite (D, G) stage. Photoactivated cells are revealed with an anti-Fluorescein antibody (red). The somitic expression of *myod*, *deltaC (dlc)* and expression of *shh* in notochord visualized in blue provides landmarks and also staging information. The labeled cell population undergoes strong extension from head to tail in control embryos (C), while rostral extension of the labeled cell population is suppressed in Apln misexpressing embryos (F). In control embryos (D, E), the anterior labeled cell population overlaps with *cmlc2* expressing cardiac precursors, whereas in Apelin misexpressing embryos (G) rostral extension of the labeled cell population is suppressed and *cmlc2* experssion missing, and in Apelin morphants (H, 10ng $MO^{apln\text{-}spl}$), labeled cells are distributed discontinuously in the heart field region and outside the stripe. (I-L) At 2 somite stage in the Apelin morphants (J), labeled anterior mesoderm cells are positioned much further laterally and somites are broadened compared to control embryos (I). (K) Depicts the relative position within the somite of mesodermal cell populations labeled by photoactivation 95° from dorsal at the onset of gastrulation in Apelin morphant (red stripe) and uninjected control embryo (blue stripe). The somite width was normalized between the control and Apelin morphant embryos. (L) Quantification of the relative position of the labeled cell populations at the first somite, demonstrating the labeled cell array was positioned in more lateral somite region of Apelin morphants compared to control embryos. 5 uninjected control embryos (0.721 of the normalized somite, sd=±0.0464) and 9 morphants embryos (0.854 of the normalized somite, sd=±0.1029) were analyzed at 10.7 hpf. Error bars depict standard deviation. ∗, $P<0.001$. Scale bar represents 200 μm (B, C, I). Vegetal pole (#). Dorsal (D). Ventral (v).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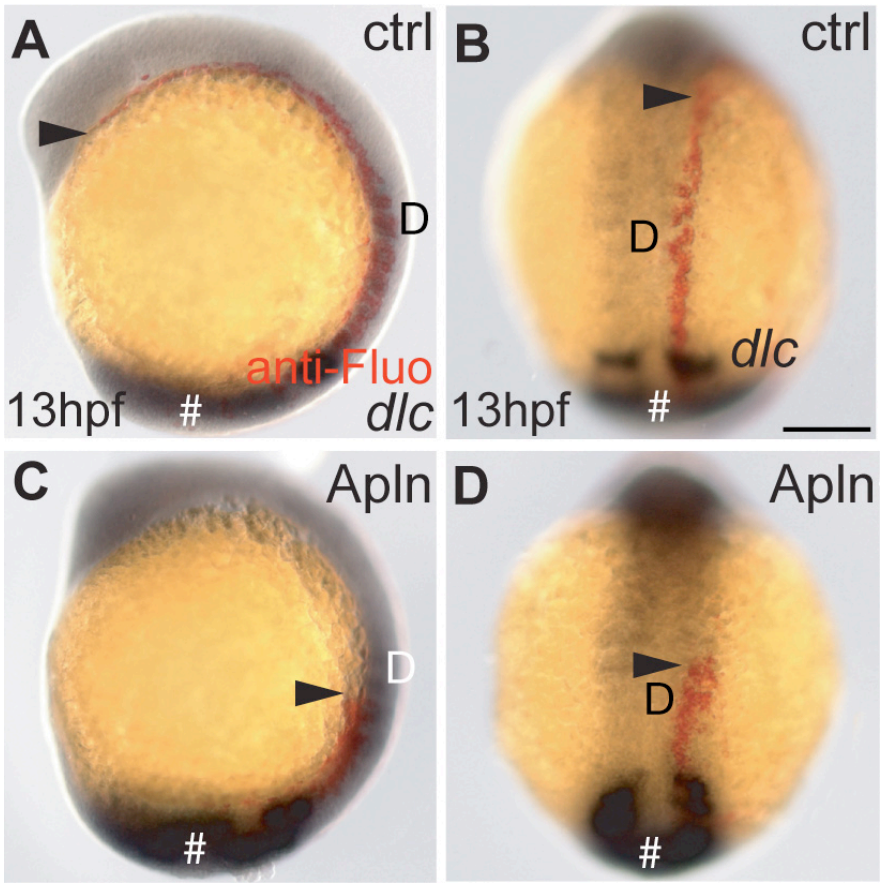


Figure S3. Cell movement defects in Apelin overexpressing embryos.
(A, C) Lateral views; (B, D) dorsal views show uninjected control embryos (A, B) and embryos ubiquitously expressing Apelin (C, D) at 8 somite stage. Cells with photoactivated Fluorescein are revealed with an anti-FITC antibody and Fast Red. The presomitic expression domains of *deltaC* (*dlc*) provide landmarks and also staging information. In control (A, B), the labeled cell population converged towards the midline and extended from the end of the tail to the most anterior part of the embryo forming a fine stripe (arrowhead). In contrast, labeled cells in embryos injected with 10 pg *apln* RNA (C, D) reached the same position within the tail but failed to extend anteriorly (arrowhead). Vegetal pole (#). Dorsal (D). Scale bar represents 200 μm (B).

stripes formed by the labeled cells extending anterior to the 8th somite was significantly shorter than in controls (Figure S3C, 310.2 ± 51.3 μm; n=15; $p<0.0001$), revealing an extension defect. We interpret these fate-mapping assays to indicate that in embryos misexpressing Apelin, anterior LPM cells including the cardiac precursors, failed to move to the correct location in the embryo and consequently failed to undergo proper cell fate specification, as demonstrated by the lack of heart marker expression.

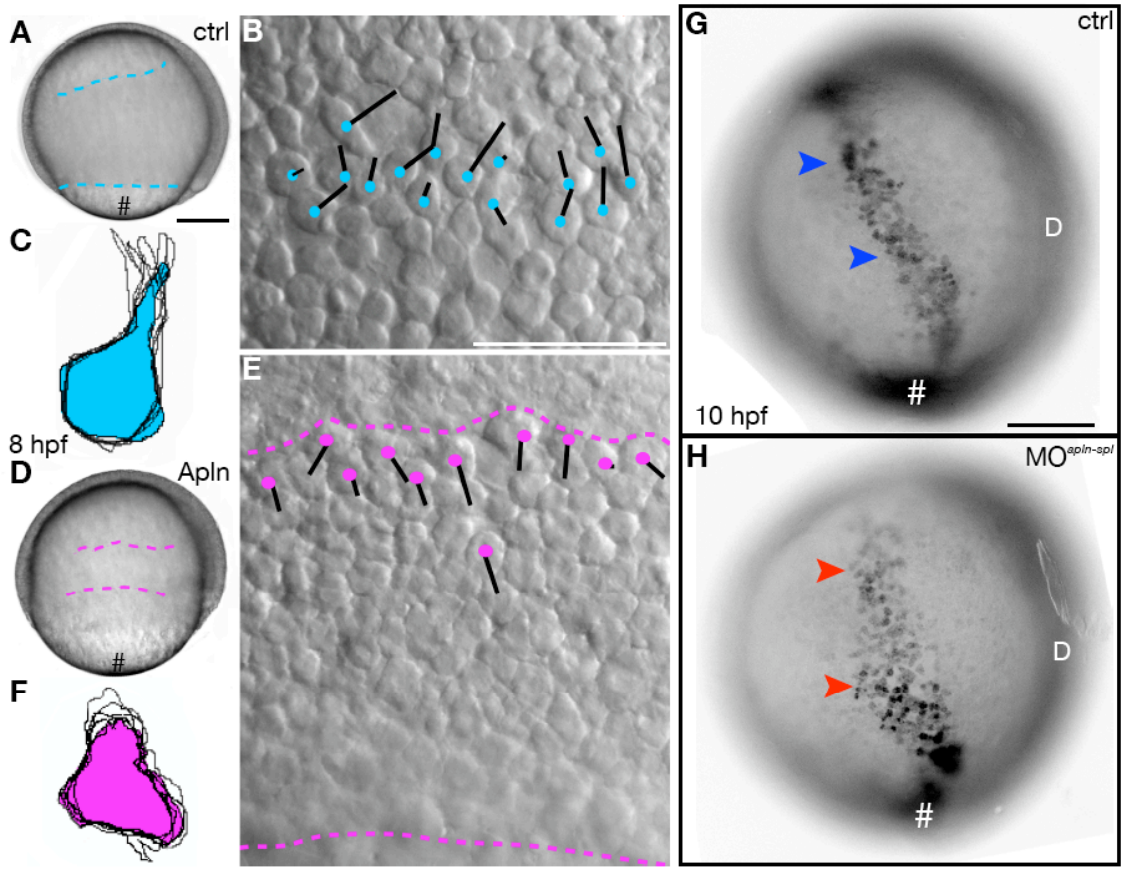
Next we asked whether heart field reduction in Apelin-deficient embryos was associated with defective cell movements. As above, in gastrulae depleted of Apelin by MO$^{apln\text{-}spl}$, lateral mesodermal cell populations were labeled by photoactivation of caged fluorescein (Figures 6A, B). By early segmentation, these cells formed stripes that extended from the head to the tail as in control experiments (Figures 6I, J). Analysis of tissue specific markers (*shh*, *deltaC*) revealed mediolaterally broadened notochord and somites in Apelin morphants, consistent with mild convergence defects of all mesodermal tissues. Strikingly, the anterior portion of the labeled cell arrays was positioned much further laterally from the midline in the Apelin morphants compared to control embryos (Figures 6I-L). This effect was quantified at the first somite, where the labeled cell array was positioned in a more lateral somite region than in control embryos (Figures 6K, L). This result indicates that convergence movements of the lateral mesoderm cells are more severely compromised than movements of other mesodermal tissues, revealing a specific requirement for Apelin in convergence movements of anterior LPM.

By midsegmentation the labeled cell arrays converged towards the midline in morphants (n=10/10, Figure 6H; and MO$^{apln\text{-}atg}$ not shown), similar to control embryos (n=8/8; Figure 6E). Notably, in the region of the heart field these cell arrays were discontinuous and broader in contrast to continuous and narrow stripes in the heart fields of control embryos (Figures 6E, H). Moreover, in Apelin morphants some labeled cells were found in ectopic positions on both sides of the array, even on the opposite side of the midline (n=9/10, Figure 6H), in contrast to control embryos (0/8). Together, these studies demonstrate that both excess and deficit of Apelin impairs migration of the anterior LPM cells during late gastrulation.

Abnormal cell movement behaviors in gastrulae with excess and deficit of Apelin

To determine whether the inhibited anterior movements of heart precursors in Apelin overexpressing embryos were associated with abnormal cell behaviors, we carried out time-lapse analyses at midgastrulation stages. Consistent with gene expression and cell tracing analyses (Figures 2M, N; 6C, D, F, G; S3), the width of the mesendoderm, from the anterior edge to margin, was reduced, confirming that both the epibolic and anterior mesendoderm movements were inhibited (Figures 7A, D and Movie S1). Cell tracking further demonstrated that the anteriorward migration of mesendodermal cells was impaired (Figures 7B, E and Movie S1) and revealed the associated cellular defects. In normally developing control embryos injected with synthetic RNA

Figure 7. Time-lapse data reveal abnormal cell behaviors of lateral mesoderm cells in embryos with excess and deficit of Apelin. Time-lapse data at midgastrula stages from control *apln∆13* RNA injected (A, B, C) and *apln* RNA injected (D, E, F) embryos. (A, D) In lateral views, anterior and posterior edges of the lateral mesendoderm are marked with dotted lines. (B, E) Net paths of mesodermal cells over 36 minutes, from embryos depicted in (A) and (D) respectively. (C, F) Mesodermal cells in control Apelin∆13 expressing gastrulae exhibit an elongated pear-shape with one predominant lamellae (C), whereas Apelin overexpressing mesodermal cells are rounder and form blebs (F). Drawings show outlines of cells at 10 second intervals for 1 minute. (G, H) Labeled lateral mesodermal cells from control (G) and MO$^{apln\text{-spl}}$ injected siblings (H) at late gastrulation stages show disrupted cell movements when *apln* is depleted. Cell arrays are more dispersed (red arrowheads) in *apln* morphant embryos (n=2) than those in uninjected control embryos (blue arrowheads) (n=2). (I) Cell polarity defects in Apelin overexpressing embryos. Lateral mesodermal cells in Apelin overexpressing gastrulae exhibit rounder shapes as revealed by determining the length to width ratio (LWR) of mesodermal cells at several stages during gastrulation. Each point represents the average LWR of 20 cells from one embryo. Error bars depict standard deviation. Scale bar represents 50 μm (B), and 200 μm (A, G). Vegetal pole (#). Dorsal (D).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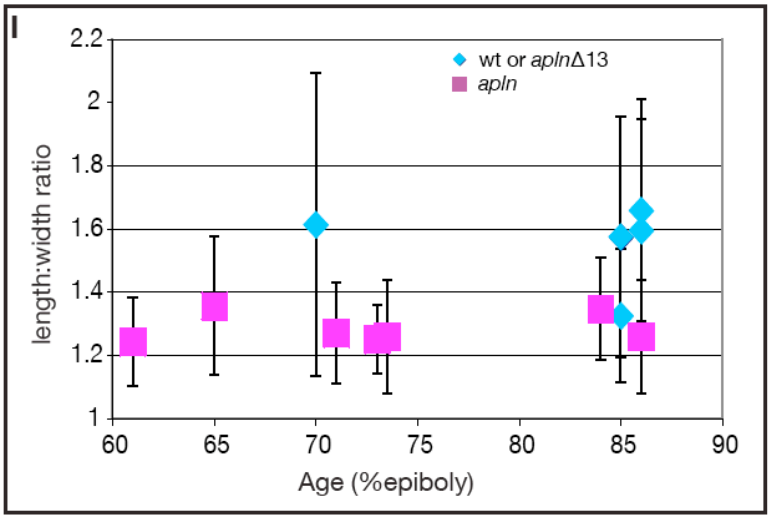
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65
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75
80
85
90
Age (%epiboly)

encoding a truncated form of Apelin (AplnΔ13) incapable of being secreted, mesendodermal cells were elongated and pear-shaped. This contrasted rounder cell shapes in Apelin-misexpressing gastrulae (Figures 7C, F, I). Mesodermal cells in the control gastrulae formed and maintained one predominant filolamellipodial protrusion at a time as expected for cells undergoing directed migration (Figure 7C; Movie S2). In contrast, cells in embryos with excess Apelin extended fewer filolamellipodia, and instead formed bleb-like protrusions (Figure7F; Movie S3). Overall, mesendodermal cells in Apelin misexpressing embryos were less able to maintain a polarized morphology typical of migrating cells (Vicente-Manzanares et al., 2005).

To ask how the loss of Apelin function effect cell movements, we carried out similar time-lapse recordings of labeled anterior LPM cells in embryos depleted of Apelin by $MO^{apln\text{-}spl}$ at late gastrulation (Movie S4-7). Whereas in the control gastrulae labeled LPM cells formed stripes that narrowed over time, in morphants the stripes appeared to narrow less or/and to fragment (Figures 7G, H, and Movie S4-7, n=2 control, n=2 *apln* morphant). Together with our fate mapping experiments (Figures 6I, J, E, H) these time-lapse analyses reveal impaired migration of LPM cells in Apelin deficient embryos.

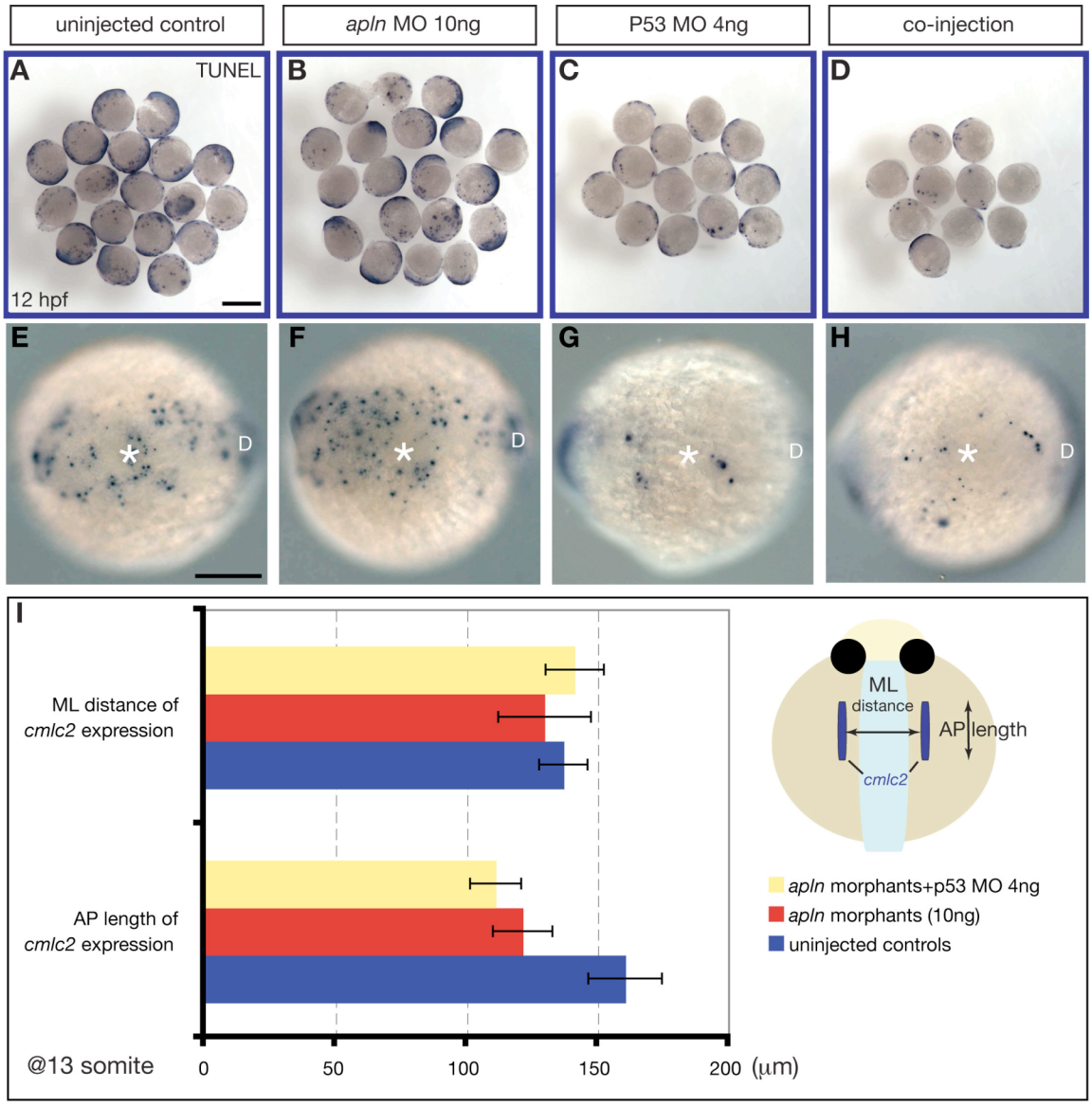
Increased cell death does not account for heart deficiency in embryos with abnormal Apelin/Agtrl1b signaling

To determine whether cell death could account for the loss of heart cells in Apelin/Agtrl1b-deficient embryos we first detected apoptotic cells using TUNEL

staining (Mizumatsu et al., 2003). We observed increased cell death in the animal half of the *apelin* and *agtrl1b* morphant embryos at the 8 somite stage, when heart field was reduced (Figures S4A, B, E, F and not shown). However, the amount of cell death did not correlate with the severity of heart loss: we observed a level of cell death in *agtrl1b* morphants (where heart was strongly reduced) that was comparable to *apelin* morphants (with much milder reduction of the heart field, Figure S4 and not shown). Second, we inhibited apoptosis by co-injecting a MO against p53 (Campbell et al., 2006) along with $MO^{apln\text{-}spl}$. While this significantly reduced cell death in both control and *apelin* morphant embryos, it did not suppress deficiency of heart precursors, as assayed by *cmlc2* expression at 15.5 hpf and 17 hpf (Figures 4L, M; S4B, D, F, H, I). These results argue against the notion that the reduction of heart field in Apelin-Agtrl1b-deficient embryos is primarily due to increased cell death. However, we cannot exclude the possibility that effects on survival may also contribute to the phenotype observed in embryos with reduced Apelin/Agtrl1b signaling.

We addressed further cell fate specification, by monitoring the earliest cardiac fates in Apelin overexpressing embryos, using *hand2, gata5* or *tbx1*. We found that these genes exhibited in the anterior LPM reduced and patchy expression, which correlated with non-cardiac cell fate populations (Figure S2 and not shown). These results indicate that excess Apelin signaling inhibits heart fate, either by directly impairing fate specification of heart precursors or indirectly by strongly impairing their migration and disrupting inductive interactions along the normal route.

Figure S4. The reduction of heart field in Apelin-Agtrl1b-deficient embryos is not due to increased cell death. TUNEL staining assay shows an increased cell death in the animal half of the *apelin* morphant embryos (B and not shown) at the 5 somite stage, compared with uninjected controls. Injection of p53 MO (4ng) alone strongly suppressed apoptosis in control embryos (C) and in *apln* morphants (D) as well. (E-F) Show higher magnification images from the animal pole view of individual representative embryos from (A-D). (I) Measurement of the anteroposterior length and the mediolateral distance of *cmlc2* expression in control, *apln* morphant and $MO^{apln\text{-}spl}$ plus MO^{p53} coinjected embryos at 13 somite stage. Suppressing cell death by injecting P53 morpholino did not rescue the heart defects due to the loss of Apelin function. Animal pole (★). Dorsal (D). AP, anteroposterior. ML, mediolateral. Each point represents the average measurement of 10 embryos. Error bars depict standard deviation. Scale bar represents 200 μm (E) and 600 μm (A).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P53 MO 4ng
co-injection
A
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12 hpf
B
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D
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ML distance of cmlc2 expression
AP length of cmlc2 expression
@13 somite
0
50
100
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(μm)
ML distance
AP length
cmlc2
apln morphants+p53 MO 4ng
apln morphants (10ng)
uninjected controls

Discussion

Here we provided several lines of evidence that gastrulation movements of anterior LPM and heart precursors in particular are highly sensitive to the level and distribution of Apelin and Agtrl1b expression. Moreover, epistasis experiments and the observation that Apelin and Agtrl1b have synergistic effects on zebrafish development in co-expression experiments, provide strong support for the notion that during zebrafish gastrulation Apelin functions upstream of Agtrl1b receptor, likely as its specific ligand.

How does Apelin-Agtrl1b signaling regulate movements of cardiac progenitors? Our analyses suggest that Apelin does not simply act as a chemoattractant or chemorepellant. Based on the following observations it is tempting to speculate that Apelin might have concentration dependent effects on cardiac precursor cell movements. First, cardiac precursors converge towards the *apln*-expressing midline during gastrulation, but stop short of reaching the midline to form bilateral fields at early segmentation (Keegan et al., 2004). We observed a delayed convergence of the LPM during gastrulation in Apelin deficient embryos (Figures 6I-L), and more dispersed and ectopic distribution of anterior LPM cells, including heart precursors at mid-segmentation (Figures 6E, H), whereas global overexpression of Apelin, strongly inhibited some of the gastrulation movements of mesodermal cells. In this scenario Apelin emanating from the midline would initially attract heart precursors until they moved near the midline to experience higher and inhibitory concentration of Apelin. It is also possible that Apelin-Agtrl1b signalling has a permissive role in cardiac progenitor

migration during gastrulation, as suggested for S1P-Miles apart GPCR signaling that is essential for migration of bilateral heart primordia to the midline during segmentation (Kupperman et al., 2000). Other roles of Apelin such as promoting adhesion of cardiac precursors to each other or to substratum, as they coalesce into the bilateral heart primordia, are also possible (Hashimoto et al., 2005).

That the convergence defect in *apln* morphants of the labeled anterior LPM cells, except the heart precursors, was corrected during segmentation (Figure 6J, K), is likely due to redundant signals guiding these cells. Indeed, *silberblick (wnt11)* mutants that manifest severe convergence and extension defects at late gastrulation, acquire a more normal body elongation by segmentation stages due to expression of *pipetail/wnt5* gene with overlapping activity (Heisenberg et al., 1996; Kilian et al., 2003). The persistent movement defect of heart precursors in embryos with excess or deficit of Apelin suggests that these cells are particularly sensitive to the level and distribution of Apelin. This enhanced sensitivity of heart precursors to Apelin/Agtrl1b signalling is puzzling given that Agtrl1b is expressed broadly in the anterior LPM and ventroposterior mesoderm during late gastrulation (Figure 1). However, expression of a GPCR beyond its target tissue has been previously reported. The GPCR CXCR-4, which guides the migration of primordial germ cells, is expressed throughout the mesoderm at the onset of gastrulation, yet the loss of CXCR-4 function disrupts only the migration of germ cells without interfering with gastrulation movements of mesodermal cells (Doitsidou et al., 2002; Knaut et al., 2003).

Together, our results reveal a requirement for G-protein coupled receptor Agtrl1b and its ligand Apelin in heart field formation, through regulation of convergence and extension gastrulation movements of cardiac precursors in zebrafish. Significantly and in contrast to global regulators of gastrulation movements, such as non-canonical Wnt signaling, prostaglandins, and Stat3 (Keller, 2005; Solnica-Krezel, 2005), Apelin signaling has a more restricted role in regulating movements of the anterior LPM cells and heart precursors in particular. We speculate that the Apelin-Agtrl1b axis provides just the first example of GPCRs regulating gastrulation movements of defined cell populations to form organ rudiments during vertebrate embryogenesis.

Experimental Procedures

Zebrafish husbandry: Zebrafish (*Danio rerio*) were maintained as described previously (Solnica-Krezel et al., 1994). Embryos were staged according to (Kimmel et al., 1995).

In situ hybridization, immunohistochemistry and histology: Single and double color whole mount *in situ* hybridization was performed essentially as described previously (Thisse et al., 1993); BM Purple (Roche) and INT/BCIP (175 µg/ml; Roche) were used as alkaline phosphatase substrates. The following molecular markers were used: *nkx2.5*, *cmlc2, vmhc*; for original references, see

(Yelon and Stainier, 1999), and *krox20*, *shh*, *dlx3b*, *dlc*, *ntl*, *myod*; for original references, see (Sepich and Solnica-Krezel, 2005).

Lineage tracing: Embryos were microinjected at the 1-cell stage with *agtrl1b* or *apln* synthetic capped RNA (Marlow et al., 1998) or an *agtrl1b*, or *apln*-specific MOs (MO^{agtrl1b}, 5'-CAGAGAAGTTGTTTGTCATGTGCTC-3' (CV109234); MO$^{apln\text{-}spl}$, 5'- AACAGCCGTCACGCTCCCGACTTAC -3' (DQ062434); MO$^{apln\text{-}atg}$, 5'-TTCTGCTCTCCCCTCCGTTTCCCTG -3' (DQ062434); Open Biosystems). A mutant form of *agtrl1b* (*agtrl1b*$^{MO\text{-}mut}$), predicted to be unable to bind MO^{agtrl1b1} was constructed using the following primers:
forward (5' - GGAATGAATGCCATGGACAAC - 3');
reverse (5' - CCAATTCTGCGTCACCCTTC - 3'). In co-injection experiments each reagent was microinjected independently at the 1-cell stage. Injection and photoactivation of anionic dextran DMNB caged fluorescein (Molecular Probes, D-3310) was performed as described (Sepich et al., 2000; Sepich and Solnica-Krezel, 2005).

Microscopy: Embryos stained by whole mount *in situ* hybridization were mounted in 80% glycerol/PBT and photographed using a Zeiss Axiophot microscope and an Axiocam digital camera. Live embryos were anesthetized if needed and mounted in 1.5% or 2.5% methylcellulose. Images were made using Photoshop and Illustrator software (Adobe).

Time-lapse analysis: Nomarski time-lapse images were collected as described (Myers et al., 2002). Multi-focal plane recordings of the lateral mesoderm (90° from the dorsal midline) were collected from 60% to 95% epiboly at 5- or 60-s intervals using a 40x or 20x objective respectively on an Axiovert200M microscope (Carl Zeiss MicroImaging) with a Retiga EX*i* camera (Q Imaging). Fluorescent time-lapse embryos were mounted in 3% agarose wells filled with 2% methylcellulose (both in Danieau's buffer). Image collection and analysis used OpenLab software (Improvision). Additional analysis used Object-Image software (Norbert Vischer, http://simon.bio.uva.nl/object-image.html) and Excel (Microsoft).

Statistical analysis: Calculations were made in Microsoft Excel. We report mean and standard deviation, and the probability associated with Student's T-Test (with 2-tailed distribution) and two samples of unequal variance.

Acknowledgments

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Supplementary Experimental Procedures

Cloning of *agtrl1b*, *apelin* and related constructs: An EST with sequence similarity to the reported Agtrl1b proteins (Devic et al., 1999; Lee et al., 2006; O'Dowd et al., 1993) was used to isolate a zebrafish homolog by 3'/5' SMART RACE (BD Biosciences) from a 6.5 hours postfertilization (hpf) cDNA preparation using the following primers 5'-CCGCATCTCCACCTCTTGCGCCCC-3' (forward), 5'-GGGTGGTGGGTGGTCCACGGAGGG-3' (forward nested), 5'-CCATCTGTCTGTCTGAGTTCACCCCCTCC-3' (reverse) and 5'-CCCTTAAAACACCCGCCGCCCCCCTCC-3' (reverse nested). Full-length *agtrl1b* was obtained by proof-reading PCR (*Pfu*Ultra, Stratagene), cloned into the *Eco*RV site of the pGEM-T Easy vector (Promega) by using the primers 5'-GGATCCACGCGGGGACATTCTGACC-3' (forward, BamH I site) and 5'-CTCGAGATAATGCACATTTTATTCACAGAGTAC -3' (reverse, Xho I site) and used for anti-sense probe synthesis with SP6 RNA polymerase after Apa I linearization. For misexpression, the full-length cDNA (BamH I/Xho I fragment) was cloned oriented into the $pCS2^{+}$ vector and linearized with Not I, and synthesized with SP6 RNA polymerase (mMESSAGE mMACHINE, Ambion). The NCBI accession number is EF079888.

Given the conservation of the C-terminal Apelin sequence in mammals (Lee et al., 2000; Tatemoto et al., 1998), the last 12 amino acids were used for BLAST searches in the zebrafish protein and EST databases. Two ESTs were identified (CA472931 and CO924544), which differed at their 3' ends. Based on the sequence in CA472931, two primers with restriction sites were designed: forward with BamH I site (5' - CGGGATCCGAGGGGAGAGCAGAAATGAA - 3'), and

reverse with EcoR I site (5' - GGAATTCTTTCAGGCTATTGTGCTGGA - 3'). A fragment was amplified from a stage 12 somite RNA preparation by RT-PCR (*Pfu*Ultra, Stratagene) and ligated into pCS2^{+}. Capped RNA was synthesized from *Not* I linearized template using SP6 RNA polymerase (mMESSAGE mMACHINE, Ambion).

We obtained upstream and downstream sequences of *apelin* by 3'/5' SMART RACE (BD Biosciences) from a 2 days postfertilization (dpf) cDNA preparation. One 5'-UTR sequence and two different 3'-UTR sequences were isolated, matching ESTs CA472931 and CO924544, and inserted into pGEM-T-easy vector (Promega). The antisense probe matching EST CA472931 revealed the expression pattern reported in this article. RNA matching EST CO924544 sequence was expressed only after 2 dpf, and is not described here. Based on SMART-RACE sequence information, we designed two primers: forward (5' - TAGCGACTGGCAGGGAAACG - 3'; reverse: 5' - TGGACCATCTTTGTTATAGGCAGATGA - 3') for cloning *apln* matching EST CA472931. RT-PCR (*Pfu*Ultra polymerase, Stratagene) products from 12-somite stage total RNA preparation were ligated into pGEM-T-easy vector (Promega) and used for anti-sense probe synthesis with SP6 RNA polymerase after *SacII* linearization. The NCBI accession number is DQ062434.

To generate a control construct lacking N-terminal signal domain for secretion but encoding the last 13 C-terminal amino acids of Apelin (PRPRLSHKGPMPF), two synthetic short complimentary oligonucleotides encoding the peptide sequences were obtained for *apln* full-length sequence.

The forward (5'-CATGGGACCTCGACCCCGCCTCTCCCATAAGGGGCCCATGCCATTCTAGA-3') and reverse (5'-GATCTCTAGAATGGCATGGGCCCCTTATGGGAGAGGCGGGGT CGAGGTCC-3') oligonucleotides were annealed, and subcloned into pCS2+ vector. All constructs were verified by DNA sequencing.

RT-PCR: Primers used for amplifying *apelin*: forward (5' - CTGGTCCAATGGCCTCCACCGAGCA - 3'); reverse (5' - TTGCCAACACGTAGCAATGGGACAAAGC - 3'); annealing temperature was 64°C, 34 cycles, 100 ng total RNA was used as a template for each developmental stage. β-*actin* was used as a loading control, and primers were: forward (5'-GGATCAGCAAGCAGGAGTACGATGAGTCTGG-3'); reverse (5'-GGAGGGCAAAGTGGTAAACGCTTCTGG-3').

Supplemental Movie Legends

Movie S1. Migration of mesendoderm is inhibited in *apln* RNA injected embryos. Same age (midgastrulation control zebrafish embryo and sibling injected with 10 ng of *apln* RNA. Images were collected with 20x objective at 1-minute intervals.

Movie S2. Cells in wild-type embryo form and maintain a single prominent filolamellopodia giving cells a pear-shaped morphology. The strong movement

toward lower right is drift of the embryo. Images were collected at 5-second intervals.

Movie S3. Cells in sibling embryo injected with 10 ng *apln* RNA are unable to form and maintain a single prominent filolamellopodia. Instead they form multiple bleb-like protrusions. Images were collected at 5-second intervals.

Movie S4-7: Convergence of lateral mesendoderm is disrupted by late gastrulation in Apelin depleted ($MO^{apln\text{-spl}}$; and $MO^{apln\text{-atg}}$ not shown) embryos. Lateral mesoderm was labelled by photo-activation of caged Fluorescein at 95 degrees from dorsal at the lateral margin. Images were collected with a 10x objective at 1 minute intervals.

Movie S4. Convergence of labeled mesoderm in a wild-type embryo at starting at YPC stage for 20 minutes. In addition to converging toward the midline, the array of fluorescently labeled cells narrows.

Movie S5. Convergence of labeled mesoderm in a wild-type embryo at starting at Tailbud stage and continuing for 20 minutes.

Movie S6. Convergence of labeled mesoderm in an Apelin-depleted embryo at starting at YPC stage for 20 minutes, a short break and an additional 20 minutes.

Movie S7. Convergence of labeled mesoderm in an Apelin-depleted embryo at starting at YPC for 20 minutes.

CHAPTER III

APELIN SIGNALING REGULATES ZEBRAFISH PRIMORDIAL GERM CELL MIGRATION

Summary

Zebrafish Apelin ligand and its G-protein coupled receptor Agtrl1b are required for heart field formation during gastrulation, when Apelin signaling regulates convergence and extension movements of cardiac precursors (Scott et al., 2007; Zeng et al., 2007). Our current analyses reveal an additional developmental role for Apelin in regulating migration of zebrafish primordial germ cells (PGCs).

During development of zebrafish and many other organisms, the PGCs migrate a long distance from the regions where they are specified towards the developing gonad where they generate gametes. The SDF1a signaling via its G protein coupled receptor CXCR4b guides migration of PGCs: loss of function of SDF1a or its receptor CXCR4b results in severe defects in PGC migration in zebrafish and other vertebrates (Doitsidou et al., 2002; Knaut et al., 2003; Molyneaux et al., 2003). During gastrulation and somitogenesis stages *apelin* mRNA is expressed in the dorsal midline, while *agtrl1b* gene, encoding the Apelin receptor, is broadly expressed in the mesendoderm, where PGCs are localized. Manipulating Apelin expression by misexpression throughout the embryo or by overexpression specifically in primordial germ cells, impaired

movements of PGCs towards their target tissues. Suppressing Apelin expression by injections of antisense morpholino oligonucleotides also resulted in a similar phenotype of mis-localized PGCs. The abnormal PGC movements in these loss and gain of function scenarios are not a secondary defect resulting from altered *sdf1a* expression. Using transplantation experiments, we showed that the cells expressing Apelin in ectopic locations attracted PGCs. However, in these experiments the PGCs stopped short of Apelin overexpressing cells. Interaction between both Apelin and Sdf1a signaling was also investigated. In *odysseus* (*ody*) (-/-) mutants, which harbor a null mutation in *cxcr4b* gene (Knaut et al., 2003), the majority of ectopic PGCs aggregate in the dorsal midline where *apelin* is expressed. Interference with both signaling pathways, by injecting *apelin* MO into *ody* (-/-) mutant embryos, significantly reduced the dorsal aggregation of PGCs. Based on the preliminary data, I hypothesize that Apelin provides an additional cue for PGCs migration during gastrulation and segmentation stages, in addition to the previously discovered SDF1/CXCR4 signaling pathway. Current experiments investigate the mechanisms via which Apelin/Agtrl1b signaling influences PGC migration.

Introduction

Migration of primordial germ cells (PGCs) is a useful model system for studying the process of directional cell movement. A common observation for many species is that the germ cells are derived from regions distinct from the site where the gonad will form. Therefore, the PGCs have to migrate travel long

distances within the embryo to arrive at the future gonad (Starz-Gaiano and Lehmann, 2001; Wylie, 1999; Wylie, 2000). This process has been studied in chick, mouse, *Xenopus*, *Drosophila*, and zebrafish where modern genetic approaches, molecular biology techniques and sophisticated imaging strategies have been applied (Howard, 1998; Rongo et al., 1997; Starz-Gaiano et al., 2001; Wylie, 1999). These studies drew a general conclusion that while the PGCs migrating towards the gonadal region, they interact with different somatic structures in the developing embryo. The somatic environment can physically carry the PGCs along as part of morphogenetic movements, meanwhile they also provide directional cues to repel them from certain regions of the embryo or attract them toward intermediate and final targets (Deshpande et al., 2001; Godin et al., 1990; Jaglarz and Howard, 1995; Kuwana and Rogulska, 1999; Matsui et al., 1990; Moore et al., 1998; Starz-Gaiano et al., 2001; Weidinger et al., 1999). However, although the descriptive migration process has been studied for several decades in various model organisms, the potential molecules functioning as signaling cues that direct PGCs toward their intermediate and final targets has remained unknown.

Studying PGC migration in zebrafish offers the benefits of fast and external embryonic development, optical clarity, and availability of mutant strains and genomic tools. Also, PGC migration in zebrafish has been described in great detail from previous studies (Braat et al., 1999; Weidinger et al., 1999; Weidinger et al., 2002; Yoon et al., 1997).

Previous work from both forward and reverse genetics approaches has

demonstrated that directional migration of PGCs in zebrafish requires the function of the chemokine Sdf1a (Doitsidou et al., 2002) and its seven-transmembrane receptor Cxcr4b (Doitsidou et al., 2002; Knaut et al., 2003). Using antisense morpholino oligonucleotides (MOs) to inhibit the translation of RNAs encoding either the receptor or the ligand abrogates directional PGC migration and thus leads to ectopic localization of these cells (Doitsidou et al., 2002). Also known as Odysseus, Cxcr4b was proven to be genetically required specifically in germ cells for their chemotaxis towards Sdf1a sources (Knaut et al., 2003). Any other signaling pathways, specifically GPCR signaling involved in directed guidance of PGC migration remain to be discovered.

Apelin and its GPCR receptor Agtrl1 have been shown to regulate adult physiology, in particular cardiovascular functions, and blood vessel development (Chen et al., 2003; Ishida et al., 2004; Saint-Geniez et al., 2002; Szokodi et al., 2002; Tatemoto et al., 2001). Our recent work reported that zebrafish Apelin ligand and its G-protein coupled receptor Agtrl1b are required for heart field formation during gastrulation, when Apelin signaling regulates convergence and extension movements of cardiac precursors (Scott et al., 2007; Zeng et al., 2007). In this work, we have revealed the chemokine Apelin as a pivotal component guiding PGCs migration. We show that during gastrulation and somitogenesis stages, *apelin* mRNA is expressed in the dorsal midline, while its receptor *agtrl1b* gene is broadly expressed in the mesendoderm, where PGCs are localized. Alterations in its expression pattern lead to corresponding alterations in the migration route of the cells. Importantly, suppressing the

function of Apelin, or its seven transmembrane G protein-coupled receptor (GPCR) Agtrl1b, lead to misguided PGC migration. Taken together, these findings indicate that Apelin and its receptor Agtrl1b are important molecules directing the PGCs toward their correct location in addition to previous discovered Sdf1a/Cxcr4b signaling pathway.

Materials and Methods

Zebrafish husbandry: Zebrafish (*Danio rerio*) were maintained as described previously (Solnica-Krezel et al., 1994). Embryos were staged according to (Kimmel et al., 1995). *Tg[askopos:EGFP]* (Blaser et al., 2005) and *Tg[gsc:Gal4-VP16]* (Inbal et al., 2006) zebrafish transgenic lines have been used.

in situ **hybridization:** Whole mount in situ hybridization was performed essentially as described (Thisse et al., 1995); BM Purple (Roche) were used as blue phosphate (175 µg/ml in DMF; Fluka). The following molecular markers were used: *nanos1* (Koprunner et al., 2001); *cmlc2* (Yelon and Stainier, 1999); *sdf1a* (Doitsidou et al., 2002); *apln* (Zeng et al., 2007).

Clone *apelin-nos1*-3' UTR construct: the *apelin* ORF was fused to the 3'-UTR of *nanos-1* for the purpose of overexpressing the protein in PGCs. The coding region of Apelin was amplified by PCR using the primers 5'-GGGGTACCGGAAACGGAGGGGAGAGCAGAA-3' (forward) and 5'-

GGGGTACCGCACTCTAAGCTGTGCCTTGCT-3' (reverse). The Apelin coding region was cloned into the *gfp-nanos-1* construct replacing the *gfp* ORF.

Clone *UAS-apelin* construct: the *apelin* ORF was fused to the vector with UAS sequences (Inbal et al., 2006). The coding region of Apelin was amplified by PCR using the primers
5'- CGGGATCCCTAGCGACTGGCAGGGAAAAC -3' (forward) and 5'-AGGGTTCGAATTTCAGGCTATTGTGCTGGAATGTC -3' (reverse). The Apelin coding region was cloned into the *UAS-gfp* (Inbal et al., 2006) construct with a Kpn I restriction enzyme site replacing the *gfp* ORF.

Transplantation experiments: Genetic mosaic analyses were performed essentially as described (Yamashita et al., 2002). WT donor embryos were injected with 0.5% rhodamine-dextran (Molecular Probes) at the one-cell stage. Apelin overexpressing donor embryos were injected at the same stage with 0.5% rhodamine-dextran (Molecular Probes) and 200 pg of *apln* synthetic RNA (Zeng et al., 2007). WT host embryos were injected with 200 pg *nos1:gfp* synthetic RNA at the one-cell stage to label PGCs. Between 4 and 5 hpf, before the embryonic shield became morphologically distinct, 30–50 deep cells at the animal pole were aspirated from one WT or one Apelin expressing donor embryo using the transplantation needle. The group of donor cells was immediately transplanted into the animal pole of a host embryo to ensure that the initial positions of the WT

or Apelin expressing donor cells were similar. The host embryos were observed at 24 hpf.

Microinjection: Embryos were microinjected at the 1-cell stage with *apln* synthetic capped RNA or *apln*-specific MOs ($MO^{apln\text{-}spl}$, 5'-AACAGCCGTCACGCTCCCGACTTAC -3', DQ062434, Open Biosystems) (Zeng et al., 2007). In co-injection experiments each reagent was microinjected independently at the 1-cell stage. Injection 0.5% rhodamine-dextran (Molecular Probes) was performed as described (Sepich et al., 2000; Sepich and Solnica-Krezel, 2005).

Microscopy: Embryos stained by whole mount *in situ* hybridization were mounted in 80% glycerol/PBT and photographed using a Zeiss Axiophot microscope and an Axiocam digital camera. Live embryos were anesthetized if needed and mounted in 1.5% or 2.5% methylcellulose. Images were made using Photoshop and Illustrator software (Adobe).

Time-lapse analysis: Nomarski time-lapse images were collected as described (Myers et al., 2002). Zebrafish transgenic line *Tg[askopos:EGFP]* (Blaser et al., 2005) has been used for recognizing the location of PGCs. Fluorescent time-lapse recordings of the PGCs were collected from 70% to 90% epiboly at 1-min intervals using a 10x objective respectively on an Axiovert200M microscope (Carl Zeiss MicroImaging) with a Retiga EX*i* camera (Q Imaging). Embryos were mounted in 3% agarose wells filled with 0.8% agarose (both in Danieau's buffer).

Image collection and analysis used OpenLab software (Improvision). Additional analysis used ImageJ software and Excel (Microsoft).

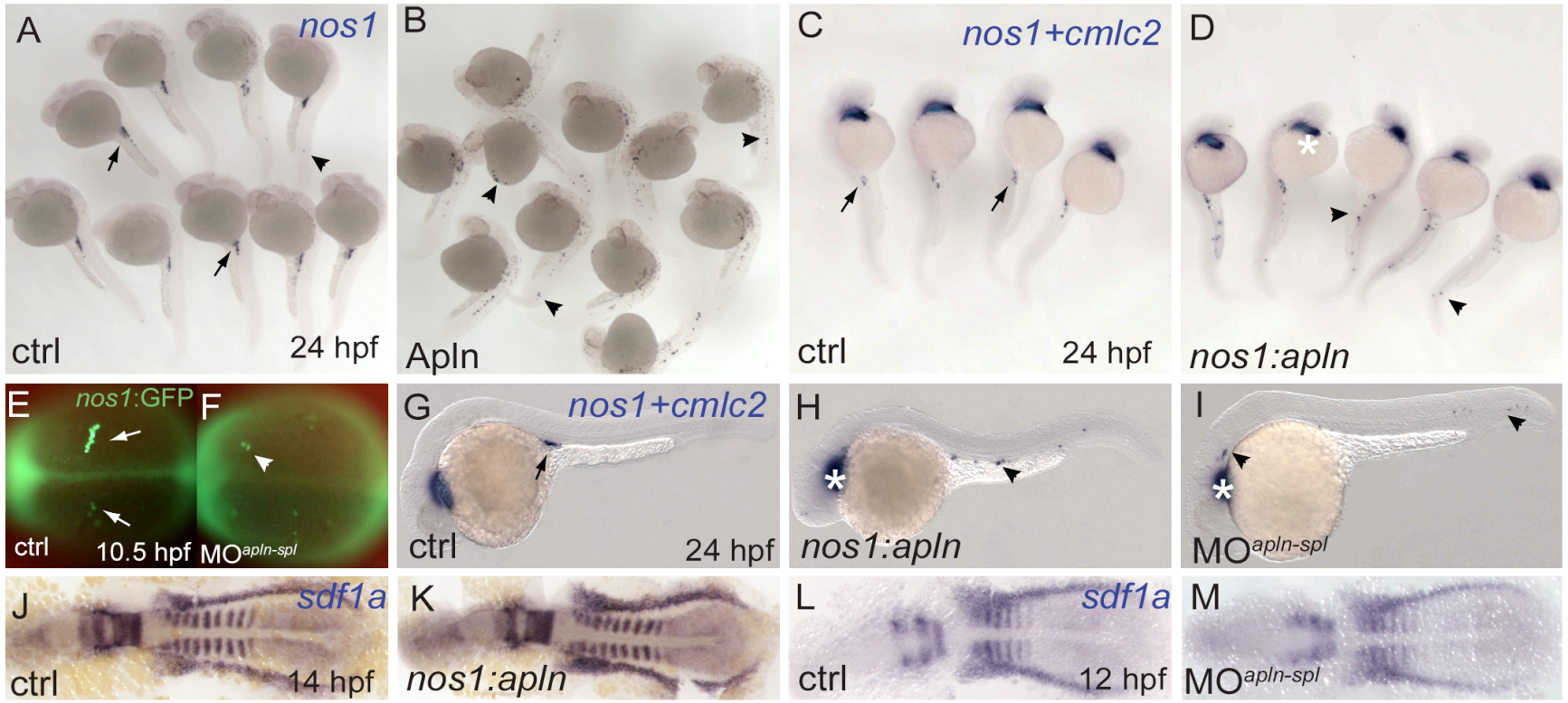
Statistical analysis: Calculations were made in Microsoft Excel. We report mean and standard error of means, and the probability associated with Student's T-Test (with 2-tailed distribution) and two samples of unequal variance.

Results

Excess or deficit of Apelin signaling influence PGCs migration

During our investigation of Apelin signaling in zebrafish gastrulation, we surveyed several cell types using cell-type specific markers, including cardiac precursor cells (*nkx2.5*, *cmlc2*, *vmhc*), somite tissues (*myod*, *dlc*), rhombomeres in the hindbrain (*krox20*), and primordial germ cells (*nanos1, nos1*). These studies revealed that Apelin signaling may regulate zebrafish primordial germ cell (PGC) migration in addition to the previously discovered Sdf1a/Cxcr4b GPCR signaling (Doitsidou et al., 2002). At 24 hpf, wild-type zebrafish PGCs, revealed by their expression of transcription factor gene *nanos1* (refs), aggregate in the trunk region where the future gonad will develop (Fig. 1A, C, G, arrows). Very few PGCs were found in other parts of the embryonic body (Fig. 1A, arrowheads) as previously reported (Dumstrei et al., 2004; Weidinger et al., 1999). When we injected synthetic RNA encoding Apelin into zebrafish zygotes at 1-cell stage to produce ubiquitous misexpression of Apelin during gastrula stages, PGC

Figure 1. Excess or deficit of Apelin signaling affects the localization of zebrafish primordial germ cells. (A-D) Misexpression of Apelin ubiquitously (B) or only in PGCs (D, H) causes dramatically mis-localization of PGCs (arrowheads) at head, heart, yolk and posterior tail regions comparing to the normal distribution of PGCs at the future gonad in control uninjected embryos (A and C, arrows) at 24 hpf. *nos1* is used for labeling PGCs and *cmlc2* is used for labeling the heart precursors by whole mount *in situ* hybridization. In wild-type embryos, ectopic localization of pGCs has been observed (A, arrowhead). The formation of heart in embryos misexpressing Apelin only PGCs is normal (D and H, star). (E, F, G, I) Loss of Apelin function by morpholino (MO) injection causes a similar phenotype with mis-localized PGCs (arrowheads) in the morphant embryos at 1 somite (F) and 24 hpf (I) stages comparing to the normal distribution of PGCs in the control embryos (E and G, arrows). (E and F) The PGCs are recognized by the expression of injected *nos1:GFP* RNA. (G-I) The PGCs are labeled by *in situ* hybridization of *nos1* expression. *cmlc2* expression (I, star) is mildly reduced in *apelin* splicing MO injected embryos compared to control siblings (G). (J-M) *sdf1a* expression is unchanged in embryos misexpresing Apelin only in PGCs (K, 14 hpf) or in *apelin* morphants (M, 12 hpf) compared to control embryos (J and L).

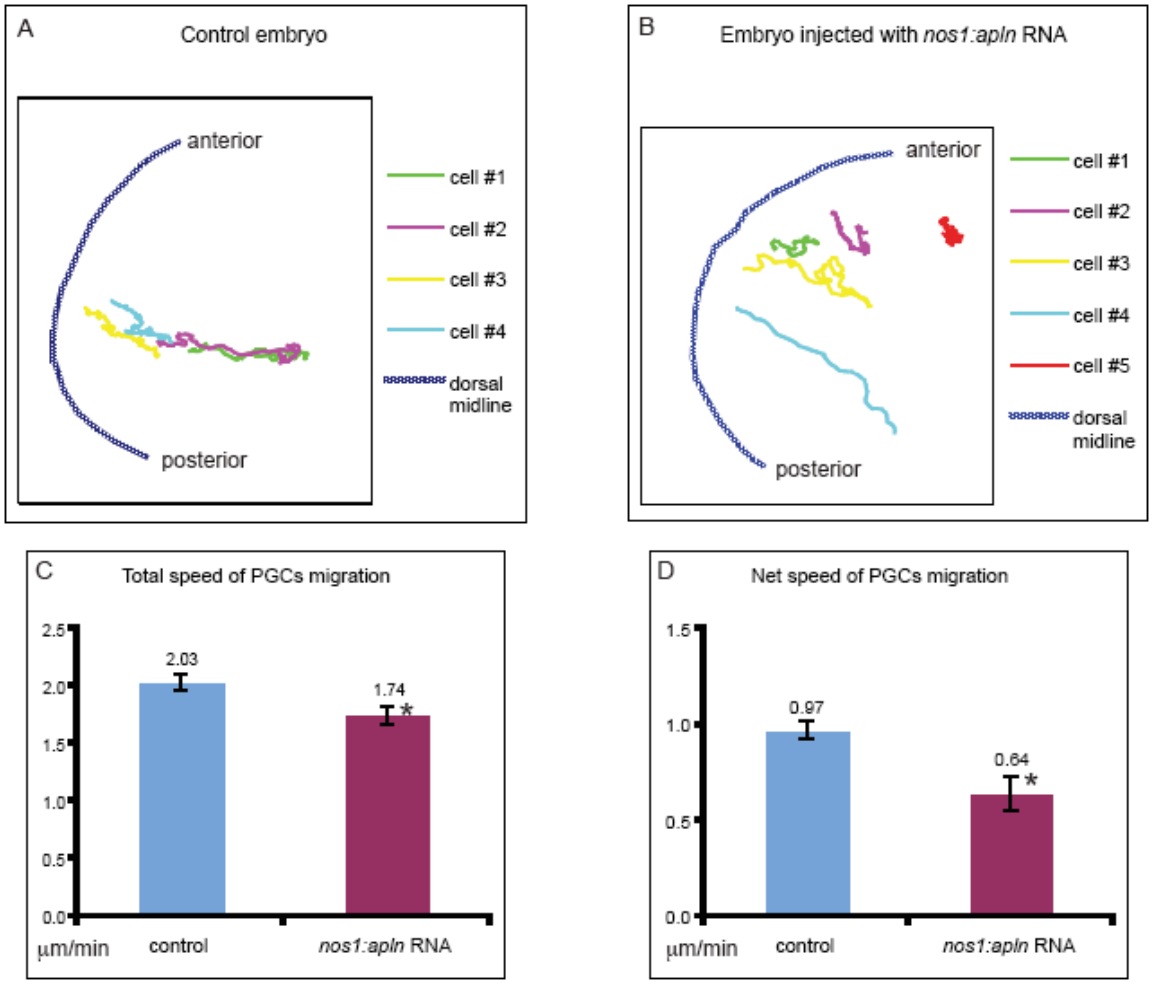

A
nos1
ctrl
24 hpf
B
Apln
C
nos1+cmlc2
ctrl
24 hpf
D
nos1:apln
E
nos1:GFP
F
ctrl
10.5 hpf
MOapln-spl
G
nos1+cmlc2
ctrl
24 hpf
H
nos1:apln
I
MOapln-spl
J
sdf1a
ctrl
14 hpf
K
nos1:apln
L
sdf1a
ctrl
12 hpf
M
MOapln-spl

localization was dramatically affected. Many ectopic PGCs were detected in the head, heart, yolk and posterior tail regions by *nos1* staining in every injected embryo (100%, n=58, Fig. 1B, arrowheads). Previously, we and others showed that ubiquitously misexpressed Apelin caused migration defects in many tissues including the anterior lateral plate mesoderm (Scott et al., 2007; Zeng et al., 2007). Therefore, the PGCs migration defects described above could be secondary due to the defects of specification, patterning or movements in other tissues. In order to test if Apelin could specifically influence the migration of PGCs, we aimed to misexpress Apelin only in the PGCs. To achieve this, we took advantage of the fact that *nos1* 3'-UTR sequences are able to stabilize an upstream RNA only in PGCs (Doitsidou et al., 2002). *nos1*:*gfp* synthetic RNA, in which GFP coding sequence is placed upstream of *nos1* 3'-UTR, has been a useful tool for specific labeling of PGCs by GFP expression (Doitsidou et al., 2002; Dumstrei et al., 2004; Weidinger et al., 2002). We replaced GFP coding sequence in the *nos1*:*gfp* construct with cDNA sequence encoding a functional Apelin peptide. The conjugated *nos1:apelin* construct, when injected into 1-cell zebrafish embryos, should be stabilized in PGCs, leading to specific misexpression of Apelin in these cells. When *nos1:apelin* synthetic RNA was injected into 1-cell stage zebrafish embryos, the migration of PGCs was impaired in these embryos misexpressing Apelin only in PGCs (Fig. 1D, H, arrowheads). Moreover, as predicted for localized Apelin expression and in contrast to global Apelin misexpression, the formation of heart in these embryos was normal based on the expression of cardiac precursor marker *cmlc2* at 24 hpf (Fig. 1D, H, star).

We next asked whether overexpression of Apelin in PGCs had any affect on the expression of *sdf1a/cxcr4b*, which are known to specifically influence the migration PGCs. We found that the *sdf1a* expression pattern in these embryos was unchanged (Fig. 1J, K). These observations support the notion that the defects of PGCs migration in embryos misexpressing Apelin specifically in the PGCs, are not due to altered expression of Sdf1a signaling components.

Using *in situ* hybridization, I observed mis-localized PGCs in embryos with altered Apelin signaling at 24 hpf, indicating an abnormal migration of these cells. However the migratory path and cell behaviors of PGCs during the 24-hour development remained unclear. In order to have a better understanding of the abnormal migration of PGCs, we performed time-lapse analyses to trace individual PGC in both control and Apelin misexpressing embryos. In our control *Tg*[*askopos*:*GFP]* transgenic embryos, in which the regulatory upstream region of the *askopos* gene (encoding a novel nuclear protein whose RNA is expressed in the germ plasma and the PGCs) and RNA elements of *nanos1* drive GFP expression in PGCs (Dumstrei et al., 2004), PGCs are visualized by green fluorescence. Most of them were clustered at the same anteroposterior level as the first forming somite and showed a very directed migration paths toward the dorsal midline at 7-9 hpf (Fig. 2A). In embryos injected with *nos1:apelin* synthetic RNA, most PGCs were less clustered together and moved toward the dorsal midline in a much less directional fashion (Fig. 2B). Some PGCs did not move at all and wandered around the same spot. Both the total speed and the net speed of PGCs in the gain-of-function situation were significantly reduced compared to

Figure 2. Time-lapse analyses reveal undirected paths and deceased average speed of PGCs migration in embryos misexpresing Apelin only in PGCs compared to control embryos. (A and B) Each colored line represents the migratory path of an individual PGC in embryos misexpressing Apelin only in PGCs (B) and control siblings (A) during 7 hpf to 9 hpf. The blue dotted line (A and B) represents the relative position of the embryo dorsal midline. The anteroposterior axis is indicated by “anterior” and “posterior”. (C and D) Both the total speed (C) and the net speed (D) of PGCs in the gain-of-function situation are significantly reduced compared to control siblings. The total speed is defined as the total distance (the accumulated all traveling paths) divided by time. The net speed is defined as the net distance (the displacement between the start point and the end point) divided by time. *, $P<0.05$.


A
Control embryo
anterior
posterior
cell #1
cell #2
cell #3
cell #4
dorsal midline
B
Embryo injected with nos1:apln RNA
anterior
posterior
cell #1
cell #2
cell #3
cell #4
cell #5
dorsal midline
C
Total speed of PGCs migration
2.5
2.0
1.5
1.0
0.5
0.0
2.03
1.74
*
µm/min
control
nos1:apln RNA
D
Net speed of PGCs migration
1.5
1.0
0.5
0.0
0.97
0.64
*
µm/min
control
nos1:apln RNA

control siblings (Fig. 2C, D). Thus, misexpression of Apelin in PGCs disrupted the normal migration pattern/behaviors of PGCs suggesting that excess Apelin influences this process and an appropriate expression source of Apelin is required for maintaining the normal migration route of PGCs.

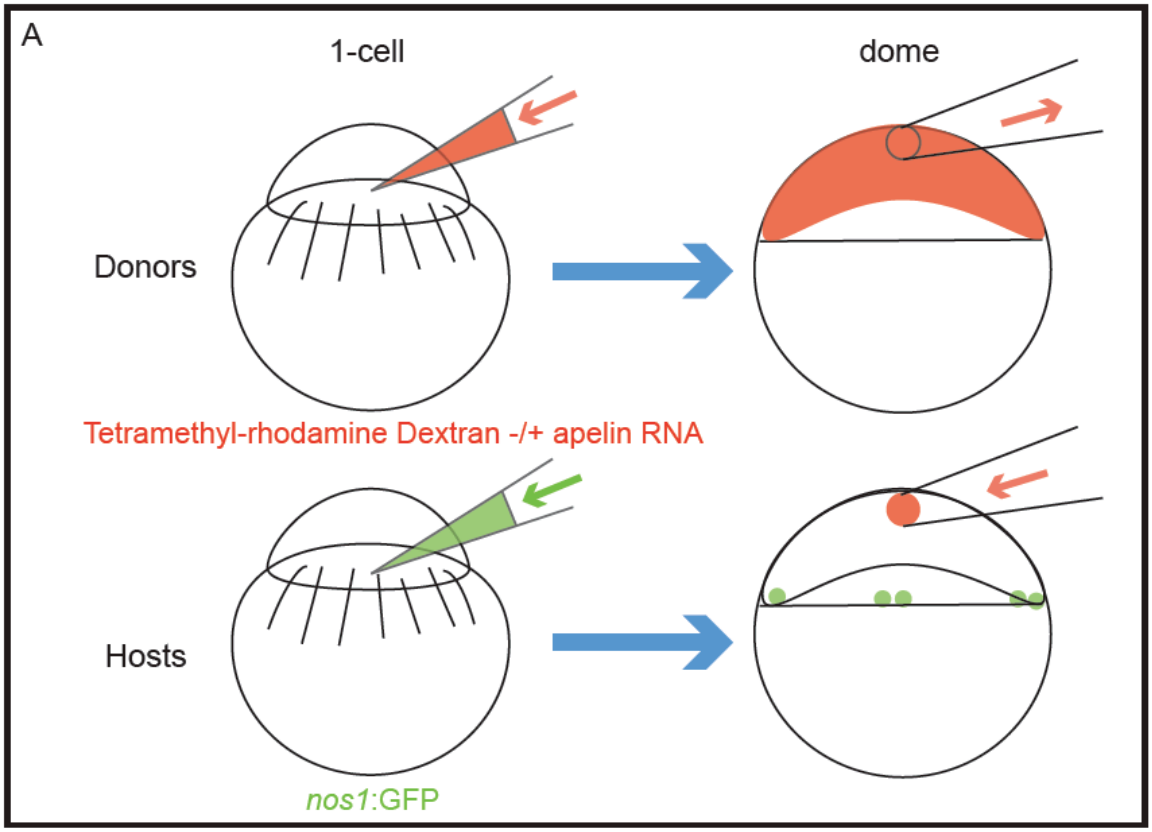
Next we asked if endogenous *apelin* is necessary for normal PGCs migration. We injected embryos with antisense morpholino oligonucleotides (Hudziak et al., 1996; Nasevicius and Ekker, 2000; Summerton and Weller, 1997) designed to interfere with splicing of *apelin* RNA and to downregulate Apelin expression level (Zeng et al., 2007). Similar to our gain-of-function study, embryos injected with $MO^{apln\text{-}spl}$ (Zeng et al., 2007) exhibited dispersed PGCs at the 1-somite stage (Fig. 1F, arrowheads) compared to control siblings, in which most of PGCs were clustered along the most anterior somites (Fig. 1E, arrows). At 24 hpf, the embryos exhibited ectopically located PGCs (Fig. 1I, arrowheads) as well as the previously described mild cardiac precursor deficit (Fig. 1I, star) (Zeng et al., 2007). The expression pattern of *sdf1a* in *apelin* morphants was examined as well, and was comparable to control siblings (Fig. 1L, M), indicating that the PGC migration defects in embryos depleted of Apelin function are not due to altered expression of Sdf1a signaling components.

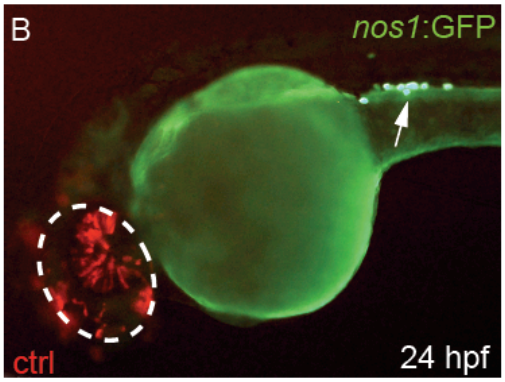
Taken together, these experiments show that Apelin activity in PGCs is important for their directional migration. This finding allows us now to follow whether Apelin acts as a chemokine-dependent mechanism to affect PGCs migration in live embryos.

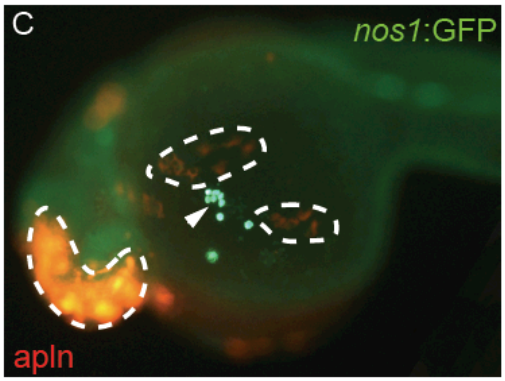
An ectopic source of Apelin attracts PGCs

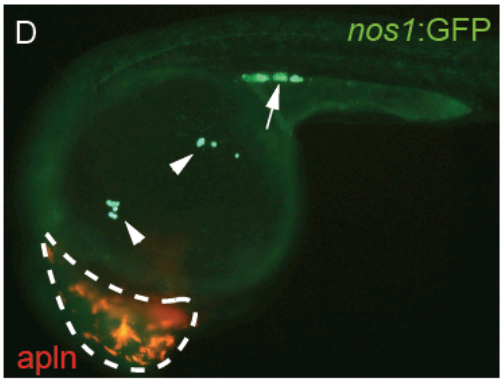
Our gain and loss-of-function analyses showed that altered Apelin signaling impairs PGCs migration. To understand the mechanism by which Apelin influences the migration process of PGCs, we wanted to provide a localized Apelin source at an ectopic position in the embryo, as a test of whether Apelin can serve as a chemoattractant for PGCs. To generate a localized Apelin source we transplanted labeled Apelin overexpressing cells into “host” embryos that had PGCs marked by green fluorescence. Specifically, we injected Tetramethyl-rhodamine Dextran lineage tracer alone or with and *apelin* synthetic RNA into donor embryos at the 1-cell stage. Host embryos were injected at the 1-cell stage with *nos1*:*gfp* synthetic RNA to label PGCs (Fig. 3A). By dome stage, blastomeres in “donor” embryos showed red fluorescence and PGCs showed green fluorescence in host embryos. We transplanted small groups of cells from the animal pole region of donors to a similar area in the host embryos (Fig. 3A). Cells were placed at the animal pole region where endogenous *apelin* is not expressed, because cells at the animal pole do not undergo C&E movements and will remain in the head region at later stages (Solnica-Krezel, 2005; Solnica-Krezel and Cooper, 2002). In control transplantation experiments, fluorescently labeled blastomeres without exogenous *apelin* were transplanted to host embryos. Transplanted embryos were raised overnight and observed under a fluorescent microscope at 24 hpf for localization of transplanted cells and labeled PGCs.

Figure 3. Ectopic Apelin expressing cells attract PGCs. (A) The procedure of transplantation experiment. Red, represents Tetramethyl-rhodamine Dextran. Green, represent *nos1:GFP* RNA. (B), control embryos. (C and D) embryos are transplanted with ectopic Apelin expressing cells. Dotted white lines indicate the area (red) of control cells with Tetramethyl-rhodamine Dextran alone (B) or Apelin expressing cells (C and D). Normally distributed PGCs (green, arrows) in control (B) and Ectopic Apelin (D) embryos. Mis-localized PGCs (green, arrowheads) are attracted to the sources of ectopic Apelin (C and D).


A
1-cell
dome
Donors
Tetramethyl-rhodamine Dextran -/+ apelin RNA
Hosts
nos1:GFP


B
nos1:GFP
ctrl
24 hpf


C
nos1:GFP
apln


D
nos1:GFP
apln

At 24 hpf in our control embryos, most donor cells, recognized by their red-fluorescence, were located in the head region as predicted (Fig. 3B, circled). Also unsurprisingly, most PGCs recognized by green-fluorescence, aggregated normally in the future gonad region. Only a few control embryos exhibited ectopic PGCs (2/19 embryos showed ectopic PGCs and there were 2±1 ectopic PGCs in each embryo) (Fig. 3B, arrows), a phenotype similar to mis-localization of PGCs in unmanipulated embryos (Weidinger et al., 1999).

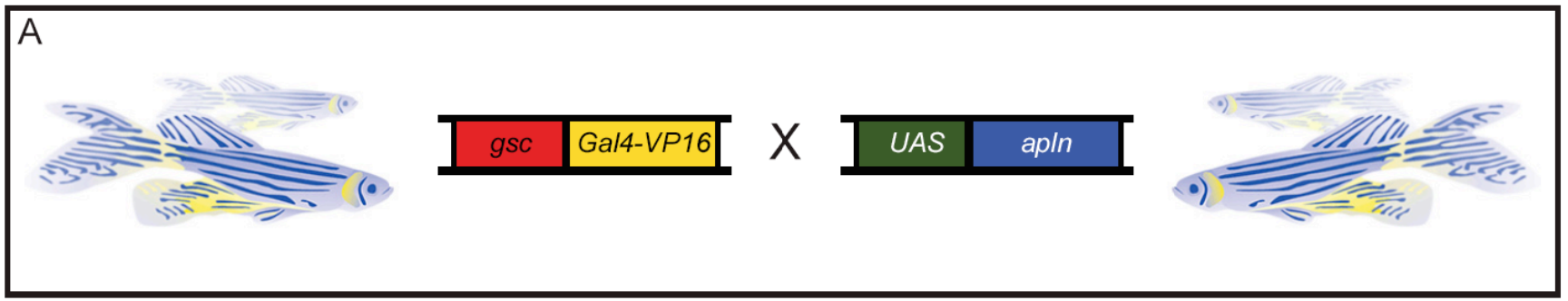
When Apelin expressing cells were present at ectopic locations at 24 hpf (Fig. 3C, D, broken white line), ectopically located PGCs were observed in the vicinity of the *apelin*-positive transplanted cells (Fig. 3C, D, arrowheads). We also noticed that some PGCs were still localized correctly at the gonad region (Fig. 3D, arrow in green). A majority (92%) of host embryos with *apelin* expressing transplanted cells showed mis-localized PGCs, and each embryo had a much larger average number of mis-localized PGCs than control embryos (36/39 embryos showed ectopic PGCs and there were ectopic 8±4 PGCs in each embryo).

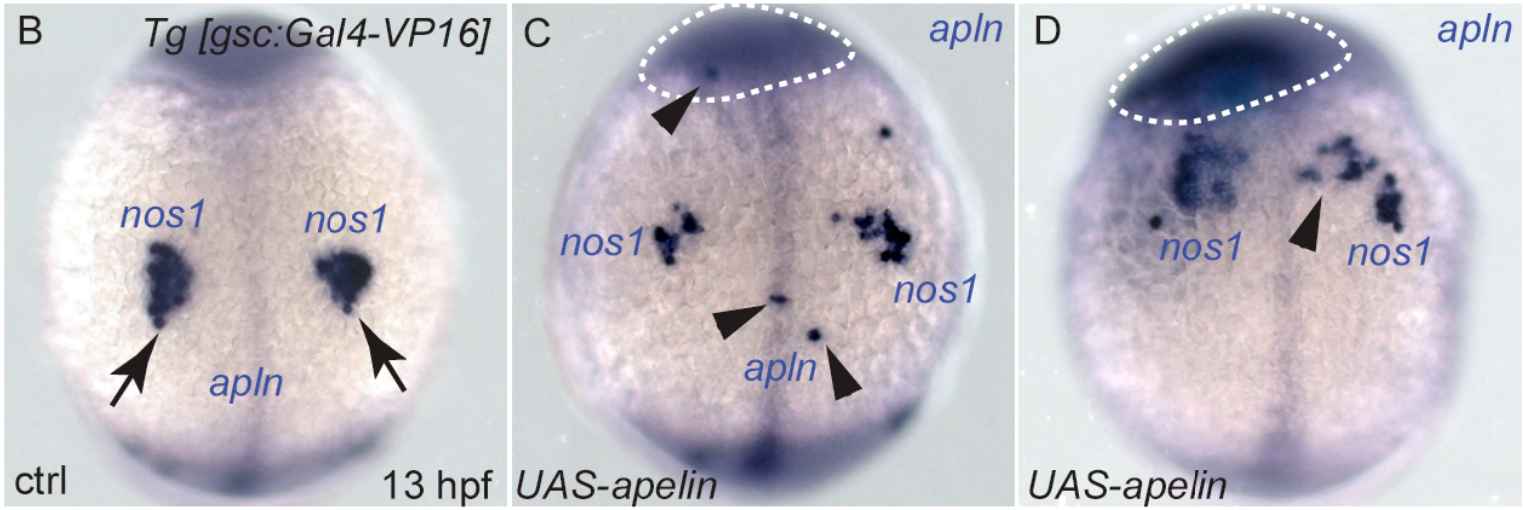
Next, we used the UAS-Gal4 system to misexpress Apelin in a tissue-specific, genetically controlled manner. The UAS-Gal4 system has been widely used in *D. melanogaster* (McGuire et al., 2004), and it consists of two transgenic lines: a Gal4 driver line and an UAS-gene line. The Gal4 transgene in the driver line produces the yeast transcription factor (Gal4) under the control of a tissue-specific promoter, and the UAS line contains a gene of interest with an upstream UAS sequence that is recognized and bound by Gal4 protein. In the presence of

both transgenes, Gal4 binds to the UAS sequence and induces the expression of the gene of interest. We used *Tg[gsc:Gal4-VP16]* transgenic fish, in which Gal4-VP16 fusion protein is expressed under the regulation of part of the *goosecoid* (*gsc*) gene promoter (Inbal et al., 2006). We chose to use Gal4-VP16, a chimera between the yeast Gal4 DNA-binding domain and the viral transactivation domain VP16 (Sadowski et al., 1988), because it is a more potent activator of transcription than Gal4 in zebrafish (Koster and Fraser, 2001). When this fish line was crossed with *Tg[UAS-GFP]* transgenic line, the next generation embryos have specifically expressed GFP in the axial mesoderm including anterior chordamesodem — the prechordal plate (Inbal et al., 2006). We generated an *UAS-apelin* construct, and we expect to see elevated *apelin* expression in the dorsal midline and the prechordal plate region in embryos that harbor both *UAS-apelin* and *gsc*:*Gal4-VP16* transgenes (Fig. 4A).

Before generating a stable *[UAS-apelin]* transgenic fish, we performed pilot DNA injection experiments. We injected *UAS-apelin* DNA together with synthetic RNA encoding Sleeping Beauty Transposase (Davidson et al., 2003) into *Tg[gsc:Gal4-VP16]* transgenic fish embryos at the 1-cell stage. In control embryos, endogenous *apelin* was expressed in the dorsal midline and PGCs were detected laterally on both sides of the dorsal midline at 14 hpf (10-somite stage) (Fig. 4B). As expected, when *UAS-apelin* DNA was co-injected with synthetic RNA encoding Sleeping Beauty Transposase, we observed *apelin* transcripts were mosaically and ectopically expressed in the prechordal plate and the notochord (Fig. 4C, D, white circles). Moreover, in these embryos the PGCs

Figure 4. PGCs migrate toward the ectopic expression of *apelin* activated by *gsc:Gal4-VP16* and *UAS:apln*. (A) The present of two transgenes *gsc:GAL4-VP16* and *UAS:apln* will produce ectopic *apelin* expression. (B) Control *Tg[gsc:Gal4-VP16]* embryos are expressing *nos1* in PGCs (arrows) and *apln* in the dorsal midline. (C and D) *Tg[gsc:Gal4-VP16]* embryos injected with *UAS-apln* DNA with synthetic RNA encoding Sleeping Beauty Transpose show ectopic expression of *apelin* in the prechordal plate (dotted white lines), and mis-located PGCs (C and D, arrowheads) in these embryos are close to the ectopic sources of Apelin.





(94%, n=31) (52%, n=57) (28%, n=57)

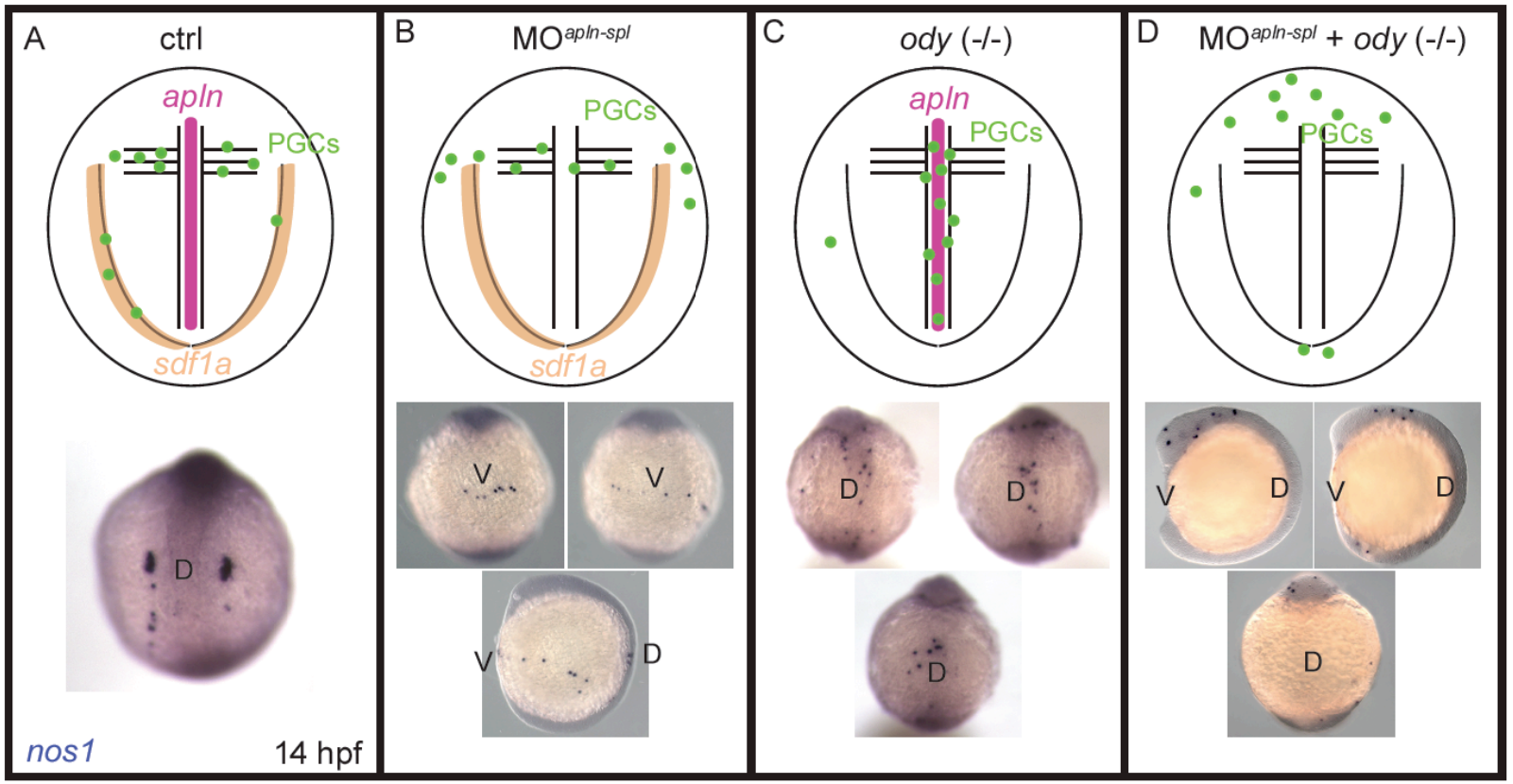
population had a tendency to be more anteriorly located toward the ectopic *apelin* sources (Fig. 4C, D, arrowheads).

Taken together, these experiments showed that the cells expressing Apelin in ectopic locations attract PGCs. But interestingly the PGCs stopped short of Apelin misexpressing cells, suggesting that Apelin might provide a concentration-dependent cue for PGC migration.

Apelin and Sdf1a signaling pathways work together to regulate PGCs migration

One of the most important outstanding questions is whether Apelin signaling interacts with previously discovered Sdf1a/Cxcr4b signaling. During mid-somitogenesis stages, *sdf1a* is expressed in the somites and intermediate mesoderm (Doitsidou et al., 2002), where PGCs are located (Fig. 5A), and *apelin* is expressed in the dorsal midline (Zeng, et al., 2007). Given these expression domains of both signaling molecules, PGCs could be under the influence of both. In *apelin* MO injected embryos, we noticed that a portion of PGCs remained in the ventral or lateral regions (68%, n=47) (Fig. 5B), suggesting the endogenous *apelin* is required for PGCs to migrate toward the dorsal region of the embryo. In *ody* homozygous mutants that lack a functional Cxcr4b receptor, the Sdf1a ligand is unable to activate downstream signaling and regulate PGC migration (Knaut et al., 2003). Previous study illustrated that Sdf1a signaling influences PGC migration very effectively. Based on these observations we hypothesized that Apelin signaling could become dominant in the embryos when Sdf1a signaling is

Figure 5. Both Apelin and Sdf1a signaling pathways are required to regulate PGCs migration. (A) wild-type control embryos, (B) wild-type embryos injected with *apelin* splicing morpholino, (C) *ody* homozygous mutant embryos, and (D) *ody* homozygous mutant embryos injected with *apelin* splicing morpholino. *nos1* is used for labeling PGCs by whole mount *in situ* hybridization. V, ventral. D, dorsal.



Ectopic PGCs	5%, 1/20	68%, 45/47	100%, 38/38	100%, 12/12
Ectopic PGCs in the midline	0, 0/20	11%, 2/47	100%, 38/38	25%, 3/12

impaired or absent. We observed that the majority of PGCs population was aggregated in the dorsal midline area where *apelin* is strongly expressed (100%, n=38) (Fig. 5C). This observation suggests Apelin attracts most of the PGCs towards the dorsal midline when Sdf1a is not present. We also injected *apelin* MO into the *ody* mutants to deplete both signaling systems. Interestingly, the aggregation of PGCs is significantly reduced comparing to *apelin* MO injected wild-type embryos (75%, n=12) (Fig. 5D). The above results suggest that Apelin and Sdf1a may regulate PGCs migration in a balanced system, and both of them work together to guide PGCs migrating correctly.

Discussion

During development of zebrafish, PGCs originate in four different positions of the blastoderm margin, randomly oriented with respect to the prospective embryonic axis (Weidinger et al., 1999), and they subsequently migrate a long distance from the regions where they are specified towards the developing gonad where they generate gametes (Starz-Gaiano and Lehmann, 2001; Wylie, 2000). During migration, zebrafish PGCs pass through somatic tissues on their way toward two clustering positions on either side of the body axis in the region where the gonad will be formed (Weidinger et al., 1999). In this study, we provide several lines of strong evidence for the involvement of the chemokine Apelin in providing directional information to the migrating cells.

First, during gastrulation and somitogenesis stages, *apelin* mRNA is expressed in the dorsal midline, while the *agtrl1b* gene, encoding the Apelin

receptor, is broadly expressed in the mesendoderm, where PGCs are localized. Hence, the expression pattern of *apelin* resides in the positions toward which the PGCs migrate. Also, our gain and loss-of-function analyses illustrated that the PGCs migration is impaired in embryos with deficit or excess Apelin signaling. Notably, a portion of PGCs remained in the ventral or lateral part of the embryo with deficit Apelin signaling. Furthermore, we show that migration of the PGCs can be redirected toward sites of ectopically expressed Apelin. Last, we show that in *ody* (-/-) mutant embryos where Sdf1a signaling is reduced, the PGCs tend to aggregate toward the dorsal midline region where the endogenous *apelin* is expressed. Our interpretation of these findings is that Apelin acts as a natural chemoattractant expressing in the somatic tissues for zebrafish PGCs, directing migration toward bilateral clustering flanking the body axis in the region where the gonad will be formed during gastrulation and early somitogenesis stages. Furthermore, the attractive role of Apelin is balanced by Sdf1a signaling.

We also argue that previously discovered Sdf1/Cxcr4b signaling could not be the only signaling pathway that regulates the migration of zebrafish PGCs. First, the dorsal midline expression of *apelin* probably provides a perfect signal cue to guide PGCs at least some of them to direct their dorsal-ward movement during gastrulation stages, and this job seems unlikely to be fulfilled by Sdf1a/Cxcr4b signaling considering their ubiquitous expression domains during gastrulation stages (Doitsidou et al., 2002). Second, consistent with our model, Apelin has been shown to act as the chemotactic factor *per se*, directing cells toward their target. Recent work from us and others reveal an essential

developmental role for the Apelin signaling in mesodermal cell movements and regulating the migration of cardiac precursors to form the heart field during vertebrate gastrulation (Zeng et al., 2007; Scott et al., 2007). Without the endogenous Apelin in the dorsal middle, cardiac precursor cells were positioned further laterally on both sides of the embryo injected with *apelin* MOs, suggesting the migration defects of these cardiac precursor cells and also a potential attractive role of Apelin in this process (Zeng et al., 2007). Third, our results show that Apelin misguide the PGCs by expressing the ligand within them, also germ cells were redirected toward ectopic positions by applying Apelin to these positions. These experiments provided strong evidence that Apelin is sufficient to influence the migration of zebrafish PGCs. However, the fact that we did not see a physical overlapping between mislocated PGCs and ectopic Apelin positions, which has been observed in an ectopic Sdf1a condition, could be explained as follows: within these host embryos, there are still endogenous Apelin and Sdf1a present. The competition between ectopic and endogenous attracting forces may result in the observation that the PGCs stopped short of Apelin misexpressing cells instead of physically attaching to each other. Future experiments of transplanting Apelin expressing cells into embryos depleted of either or both Apelin/Sdf1a signaling will give us a clearer results to finalize our current model.

Based on these data, we propose a preliminary model for the migration process of zebrafish PGCs. During zebrafish gastrulation, the dorsal midline expressing Apelin functions as a chemoattractant to guide PGCs, which are randomly positioned in the blastoderm margin, toward the dorsal region in the

embryo. Meanwhile, even though *sdf1a* and *cxcr4b* are ubiquitously expressed in the gastrula, they don't have a directional role in regulating PGCs migration. During early somitogenesis stages, *sdf1a* start to be expressed in a much more restricted manner, including in the forming somites, and *apelin* continues to be expressed in the dorsal midline and attracts PGCs. At this point, we hypothesize that Sdf1a starts to function as a strong chemoattractant opposing Apelin's function, and attracts PGCs away from the dorsal region and prevents them migrate further towards the dorsal midline. Indeed, a ventral-ward migration process of PGCs at early somitogenesis has been previously reported, in which cells were away from the dorsal midline along the forming somites area during early somitogenesis stages (Weidinger et al., 1999). In summary, we propose that Apelin and Sdf1a regulate PGCs migration as a balanced system, and a precise coordination of both signaling pathways is needed to maintain PGCs in the correct migration route.

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

My dissertation research explored the roles of a pair of proteins, the Apelin ligand and its receptor, Agtrl1b, during zebrafish embryogenesis. Our results revealed that they functioned in cell migration during zebrafish early development, more specifically, by regulating the migration of cardiac precursor cells and primordial germ cells.

The vertebrate heart primordia arise during gastrulation, when the anterior lateral plate mesoderm undergoes convergence and extension movements to form bilateral heart fields flanking the embryo midline (Keegan et al., 2004). The mechanisms that regulate heart field formation remain largely unknown. The data described in Chapter II demonstrated that Apelin and its G-Protein Coupled Receptor, Agtrl1b, previously implicated in heart physiology, are essential for heart field formation during zebrafish gastrulation. Our data showed that the Agtrl1b receptor is expressed in the lateral plate mesoderm while the Apelin ligand is expressed in the embryo midline. Deficit and excess Apelin/Agtrl1b function, lead to reduction or complete absence of cardiac primordia. I provided several lines of evidence that Apelin-Agtrl1b controls heart field formation largely by influencing the movement of cardiac precursors during gastrulation. Based on the observations from our gain and loss-of-function studies, along with the fate-mapping experiments, gastrulation movements of anterior LPM and heart

precursors in particular are highly sensitive to the level and distribution of Apelin and Agtrl1b expression. A manuscript by Ian Scott and collaborators (Scott et al., 2007) (University of California San Francisco, USA), reporting an independent identification of Agtrl1b as an essential regulator of the formation of cardiac primordia during zebrafish gastrulation, was published in parallel with our publication (Zeng et al., 2007). Their studies arrived at very similar conclusions that Agtrl1b and Apelin regulate migration of mesodermal precursors of the myocardial lineage. Interestingly, in contrast to our reverse genetic studies they discovered a role of *agtrl1b* gene in cardiac field formation via a forward genetic approach. In a screen for mutations affecting heart formation, they identified and characterized the phenotype of embryos carrying a germline mutation in *agtrl1b* (*grinch, grn*) exhibited the defective cardiac phenotype. Also, they reported that embryos with this mutation show incomplete penetrance and variable expressivity. In addition, recent loss-of-function experiments in frog embryos have shown vascular developmental abnormalities, varying from perturbed intersomitic vessel branching to more fundamental developmental defects, including decreased numbers of endothelial cells (Cox et al., 2006; Inui et al., 2006a). However, the described severe cardiac defects in frog embryos with depletion of *agtrl1b* were interpreted as secondary to the primary endothelial effects (Inui et al., 2006a). While the studies in zebrafish documented expression of *agtrl1b* receptor in endothelial cells associated with the heart and major vessels (Scott et al., 2007; Zeng et al., 2007), they did not find evidence of disrupted vascular development in experiments where Agtrl1b or Apelin function

was inhibited. The different observations of Apelin signaling in vascular development between the frog and zebrafish may reflect diverse functions of this signaling pathway during vertebrate evolution or a fundamental difference in zebrafish adult physiology. Considering the existence of another *agtrl1* gene, *agtrl1a*, in the zebrafish genome, a further examination of *agtrl1a/b* double mutants will be required to fully assess the role of Agtrl1 signaling in zebrafish angiogenesis. Finally, it is unclear why only cardiac development is severely affected, since the receptor transcripts of *agtrl1b* are found in most mesodermal precursors. Similar observations have been made in the case of Sdf1a/Cxcr4b ligand-receptor pair, which regulates the migration of zebrafish primordial germ cells (PGCs) (see below). The PGCs appear to be very sensitive to changes in the level of Sdf1a ligand, but both Sdf1a and its receptor Cxcr4b were found in a wide variety of cell types and tissues (Doitsidou et al., 2002). Furthermore, embryos in which either Sdf1a or Cxcr4b activity was impaired did not exhibit increased lethality or obvious somatic defects, despite the widespread expression of these genes (Doitsidou et al., 2002); (Knaut et al., 2003). One possible explanation is that the expression (either RNA or protein level) of a GPCR does not necessarily reflect a competence to respond to its ligand. Additional interactions, either intracellular or extracellular, with other molecules can suppress its function when necessary, or *vice versa* synergistically increase its function. Hence, the requirement for additional components of Apelin/Agtrl1b pathway or for action of mutiple signaling pathways might partially explain why cardiac precursors are particularly sensitive to Apelin/Agtrl1b while the receptor

is so broadly expressed.

Apelin signaling via the Agtrl1 receptor to activate downstream components has been reported in mammalian systems (Lee et al., 2000; Tatemoto et al., 1998), and we investigated and validated this relationship in zebrafish as well. We reasoned that ligand and its receptor should have synergistic effects on development in coinjection experiments. Accordingly, embryos injected separately with low doses of synthetic RNAs encoding Agtrl1b or Apelin progressed through gastrulation, whereas embryos coinjected with the same doses of both RNAs underwent developmental arrest by late blastula stages, most dying by 1 dpf (Chapter II, Figures 5A-E). We also expected that Agtrl1b function should be required for excess Apelin to impair gastrulation movements. Accordingly, injections of $MO^{agtrl1b}$ significantly suppressed the epiboly delay caused by Apelin misexpression (Chapter II, Figures 5F-J). In agreement, data from the Stainier's group showed that cells stably overexpressing zebrafish Agtrl1b are responsive to human Apelin peptides, resulting in adenylylcyclase inhibition and phosphorylation of ERK and p70S6 kinase. In contrast to the wild-type gene, the grn^{s608} allele of *agtrl1b* was reported to be completely unresponsive to stimulation by Apelin in these assays (Scott et al., 2007). Therefore, by biochemical criteria, these data validate the assignment of Agtrl1b as a zebrafish ortholog of the Apelin receptor. Together, these results provide strong support for the notion that during zebrafish gastrulation, Apelin functions upstream of the Agtrl1b receptor, likely as its specific ligand. However, the non-identical cardiac phenotypes between loss of Apelin and loss of Agtrl1b

function make us to consider other possibilities. Although there are two *agtrl1* genes in zebrafish, there is only one *apelin* gene, so loss of this gene should phenocopy the *agtrl1b* depletion/mutation phenotype. The difference in phenotypes might imply then that another Agtrl1b ligand can compensate for loss of Apelin, at least in heart development.

How does Apelin-Agtrl1b signaling regulate movements of cardiac progenitors? Cardiac precursors converge towards the *apelin*-expressing midline during gastrulation, but stop short of reaching the midline to form bilateral fields flanking the midline at early segmentation (Keegan et al., 2004). We observed a delayed convergence of the LPM during gastrulation in Apelin-deficient embryos, and more dispersed and ectopic distribution of anterior LPM cells, including heart precursors in Apelin-deficient embryos at mid-segmentation (Chapter II, Figures 4E, H). It is tempting to speculate that Apelin might have concentration dependent effects on cardiac precursor cell movements. In particular, at lower concentration Apelin attracts the cardiac precursor cells and repels them at a higher concentration when they are physically closer to the source of the Apelin ligand. Hence, according to this model, Apelin emanating from the midline would initially attract heart precursors until they moved near the midline to experience higher and inhibitory concentration of Apelin. Another possibility is that there are other pathways, *e.g.* another GPCR signal, that regulate the migration of cardiac precursors in addition to Apelin/Agtrl1b. As I discussed before, studies from the Stainier group reported that *grn* mutant embryos show a variable penetrance of the cardiac deficiency phenotype. Similarly and interestingly, while targeted

deletion of the *agtrl1b* gene in mice produces marked cardiac defects, only half of mouse embryos homozygous for *agtrl1b* show the developmental cardiac phenotype. Preliminary analyses of mice lacking Apelin do not show these defects (Quertermous, 2007). These data are most consistent with the notion of signaling redundancy, in which an alternative pathway can functionally compensate for loss of the Apelin-Agtrl1b pathway. While this is likely another GPCR pathway, its identity remains unknown, and the subject for further study. In this case, these cardiac cells are exposed to multiple regulatory cues, and their behaviors should be considered as a reflection of accumulated inputs in the embryonic organism. In fact, as previously introduced in Chapter I, some signaling pathways that affect convergence & extension movements of the three germ layers, such as non-canonical Wnt, BMP signaling, also consequently secondarily affect the movements of heart progenitors (Schneider and Mercola, 2001; Schultheiss et al., 1997). Therefore, I hypothesize that Apelin might only have a simple chemoattractive role in zebrafish cardiac cell movement and the repulsion influence on these cells could be from another interacting factor. What I would like to emphasize is that both hypotheses are not mutually exclusive. Other roles of Apelin such as promoting adhesion of cardiac precursors to each other or to substratum, as they coalesce into the bilateral heart primordia, are also possible. Studies from human embryonic kidney 293T cells stably expressing the mouse APJ, the homolog of Agtrl1b receptor, showed that Apelin induced Akt/PKB phosphorylation, enhanced focal adhesion kinase (FAK) phosphorylation and increased focal adhesion formation. Cell motility in

APJ/293T cells was strongly accelerated as well when Apelin was provided (Hashimoto et al., 2005).

Together, our results reveal a requirement for G-protein coupled receptor Agtrl1b and its ligand Apelin in heart field formation, through regulation of convergence and extension movements of cardiac precursors in zebrafish gastrula. Significantly and in contrast to global regulators of gastrulation movements, such as non-canonical Wnt signaling, prostaglandins, and Stat3 (Solnica-Krezel, 2005), Apelin signaling has a much more restricted role to regulating movements of the anterior LPM cells, the heart precursors in particular. Data from both groups illustrates that Agtrl1b is not required for the induction of cardiac precursors. Further, coinjection of a p53 MO, which inhibits apoptosis in zebrafish embryos (Langheinrich et al., 2002), did not alleviate either *apelin* or *agtrl1b* MO phenotypes. Hence, we concluded that Apelin-Agtrl1b signaling does not appear to be primarily required for the survival of these cardiac precursor cells. Transplantation experiments from the Stainier group also support this migration model: both *agtrl1b* morphants cells in a wild-type environment and wild-type cells in a ubiquitous *apelin* background appear to migrate toward the midline more slowly. We speculate that the Apelin-Agtrl1b axis provides just the first example of GPCRs regulating the movements of defined cell populations to form organ rudiments during vertebrate gastrulation. I anticipate this work to be of interest and significance for the field of cell movement, because it uncovers a pathway that regulates a fundamental but poorly understood process of cardiac progenitor migration to form the heart field

during gastrulation. Future studies of the intracellular events are needed to understand this process more completely.

As I argued before, one cell population in the embryo may be regulated by multiple signals. And *vice versa*, a single signaling pathway can have pleiotropic effects on development. For example, besides the newly-discovered roles of Sdf1a and its receptor Cxcr4b in the directed migration of zebrafish PGCs (Doitsidou et al., 2002; Knaut et al., 2003), the SDF1-CXCR4 pathway is also known to play roles in the chemotaxis of several cell types, such as lymphocytes (Bleul et al., 1996a; Bleul et al., 1996b), and cerebellar and hippocampal neurons (Lu et al., 2002; Zou et al., 1998). It also plays a part in several pathological situations, for example, tumor metastasis (Muller et al., 2001), and joint infiltration (Buckley et al., 2000). Consistent with this notion, our continued investigation of Apelin-Agtrl1b signaling in other cell populations yielded preliminary data suggesting that they also have an important role in regulating the migration of zebrafish primordial germ cells (PGCs).

During zebrafish development, the PGCs are derived from regions distinct from the site where the gonad will form. Therefore, the PGCs have to migrate travel long distances within the embryo to arrive at the future gonad (Raz, 2004). Previous work from both forward and reverse genetics approaches has demonstrated that directional migration of PGCs in zebrafish requires the function of the chemokine Sdf1a (Doitsidou et al., 2002) and its seven-transmembrane receptor Cxcr4b (Doitsidou et al., 2002; Knaut et al., 2003). In this study, we provide several lines of evidence for the involvement of the

chemokine Apelin in providing directional information to the migrating cells, in addition to the Sdf1a/Cxcr4b axis.

First, the spatiotemporal expression pattern of genes encoding Apelin and Agtrl1b is consistent with a role in PGC migration. During gastrulation and somitogenesis stages, *apelin* mRNA is expressed in the dorsal midline, where a portion of four PGC clusters positioned at the blastoderm margin converge towards, beginning at the shield stage (Weidinger et. al., 199). The *agtrl1b* gene encoding the Apelin receptor is broadly expressed in the mesendoderm. Especially, at the beginning of gastrulation, *agtrl1b* transcripts are restricted to the mesendoderm precursors at the blastoderm margin, where four clusters of PGC are localized. Hence, *apelin* is expressed in the embryonic structure toward which the PGCs migrate. Second, our loss- and gain-of-function analyses illustrated that the PGC's migration is impaired in embryos with deficient or excess Apelin signaling, respectively. Notably, a portion of PGCs remained in the ventral or lateral part of the embryos with deficient Apelin signalling, suggesting that the correct location and level of Apelin expression are critical for a subset of PGCs to migrate away from the ventral side of the embryo where they originate (Chapter I, Figure 3A). Furthermore, we provide two lines of evidence that migration of the PGCs can be redirected toward ectopic sites of Apelin expression, including our transplantation and genetically driving ectopic expression of Apelin under the control of a *gsc* promoter by using the UAS-Gal4 system. Similar results of ectopic Sdf1a attracting PGCs have been reported (Doitsidou et al., 2002). However, PGCs migrate toward ectopic Apelin

expressing cells but little physical overlap with them has been found. Third, we observed that in *ody* (-/-) mutant embryos, where Sdf1a-Cxcr4b signaling is absent because the *ody* mutation results in a complete loss of Cxcr4b protein function (Knaut et al., 2003), the PGCs tend to aggregate toward the dorsal midline region, where *apelin* is endogenously expressed. One interpretation of these findings is that during gastrulation and early somitogenesis stages Apelin acts as an endogenous chemoattractant that directs PGC migration toward bilateral clusters flanking the embryo midline in the region where the gonad will be formed. A further comparison of the spatiotemporal expression pattern between *apelin* and *sdf1a* suggests that the attractive role of Apelin might be balanced by Sdf1a signalling. During gastrulation and early somitogenesis stages, *apelin* is continuously expressed in the dorsal midline, whereas *sdf1a* has a much more dynamic expression profile (Doitsidou et al., 2002). Specifically, at the onset of gastrulation, *sdf1a* is expressed around the blastoderm margin with the exception of the dorsal-most aspect of the embryo, coinciding with the position where PGCs are found at this stage. Later, *sdf1a* is strongly expressed in the lateral plate mesoderm of the trunk and at the border between the head and trunk mesoderm (Chapter I, Figure 3B), where the PGCs have been mainly found at this stage. This interesting correlation between the positions of PGCs, *apelin* and *sdf1a* suggests that Apelin may serve as a guidance cue for the dorsal-ward migration of PGCs, meanwhile Sdf1a functions as a tightly associated guardian that guides PGCs and also prevent them from migrating too closely to the dorsal region.

Consequently, we argue that previously discovered Sdf1/Cxcr4b signaling is not be the only signaling pathway that regulates the migration of zebrafish PGCs. First, the dorsal midline expression of *apelin* probably provides a localized signal cue to guide PGCs or a subpopulation in their dorsal-ward movement of PGCs during gastrulation stages, and this job is unlikely fulfilled by Sdf1a/Cxcr4b signaling considering their ubiquitous expression domains during gastrulation stages (Doitsidou et al., 2002). Also, our results show that Apelin misguide the PGCs when Apelin are only misexpressed in the PGCs, and also germ cells were redirected toward ectopic Apelin expressing positions. These experiments provide strong evidence that Apelin is sufficient to influence the migration of zebrafish PGCs. However, the fact that we did not see a physical overlapping of mislocated PGCs and ectopic Apelin sources contrasts what has been observed in experiments in which an ectopic Sdf1a source was generated in the fish embryo and ectopic PGCs colocalized with these ectopic Sdf1a expressing cells (Doitsidou et al., 2002). We explain the difference as follows: within these “host” embryos, in which we have created ectopic sources of Apelin, endogenous Apelin and Sdf1a are still expressed in their normal expression patterns. The competition between ectopic and endogenous attractive cues could result in the PGCs stopping short of Apelin misexpressing cells instead of physically contacting them. Future experiments of transplanting Apelin expressing cells into embryos depleted of either or both endogenous Apelin/Agtrl1b and Sdf1a/Cxcr4b expression will give us a more definitive test of our current model.

In order to support our conclusion, additional evidence that Agtrl1b receptor is involved in the migration of PGCs should be obtained. We observed the expression of *agtrl1b* in the blastoderm margin, where PGCs are found, at the onset of gastrulation (Zeng et al., 2007). The correlation between the positions of *agtrl1b* expression and location of PGCs should be analyzed at later stages, especially during late gastrulation and early somitogenesis. Also, the migration of PGCs should be investigated in *grinch* mutant embryos harbouring an *agtrl1b* mutation (Scott et al., 2007) to ask if Agtrl1b receptor is required in this process. Furthermore, we will perform experiments to restore the activity of Agtrl1b specifically in the PGCs in embryos globally depleted of the receptor (*grinch* mutant embryos (Scott et al., 2007) or MO injection(Zeng et al., 2007)). The ability of Agtrl1b to rescue the abnormal migration phenotype would provide further support for the notion that the activity of the Agtrl1b receptor is required in the migrating germ cells themselves.

In conclusion, we propose the following working model for the migration process of zebrafish PGCs. During zebrafish gastrulation, the dorsal midline expressing Apelin functions as a chemoattractant to guide PGCs, which are randomly positioned in the blastoderm margin, toward the dorsal region in the embryo. Meanwhile, even though *sdf1a* and *cxcr4b* are broadly expressed in the gastrula, they do not have a directional role in regulating PGCs migration. During early somitogenesis stages, *sdf1a* starts to be expressed in a much more restricted manner, including in the forming somites, while *apelin* continues to be expressed in the dorsal midline, where it attracts PGCs. At this point, we

hypothesize that Sdf1a starts to function as a strong chemoattractant opposing Apelin's function, and attracts PGCs away from the dorsal region and prevents them from migrating further towards the dorsal midline. Indeed, migration of PGCs laterally and away from the dorsal midline along the forming somites area during early somitogenesis stages has been previously reported (Weidinger et al., 1999). In summary, we propose that Apelin and Sdf1a regulate PGCs migration as a balanced system, and a precise coordination of both signaling pathways is needed to maintain PGCs on the correct migration route.

Upon the completion of my Ph.D. training, there are still several remaining important open questions in this project. What is the intracellular mechanism via which Apelin regulate the migration of cardiac precursor cells and primordial germ cells? Are they the same or different? We know Apelin signaling is involved in the migratory process of both cell populations, however, it is not clear what cell properties it affects in each cell type: is the general motility or the directed cell movement of these cells affected? Does the cell adhesion change when Apelin signaling is altered? What are the downstream components of the Apelin-Agtrl1b axis? Is the same G alpha subunit involved in both cases?

Studies in cell culture and mammalian systems suggest that activation of the Apelin receptor elicits effects through multiple signaling pathways. Apelin-stimulated augmentation of extracellular acidification rates in Chinese hamster ovary cells is sensitive to pertussis toxin, and Apelin inhibits forskolin-stimulated production of cAMP, which indicates the presence of a Gi signaling cascade (Habata et al., 1999; Hashimoto et al., 2005; Hosoya et al., 2000; Masri et al.,

2006; Reaux et al., 2001). Apelin activates p70 S6 kinase, which is an important regulator of translation and cell-cycle progression, through both phosphoinositide 3-kinase and extracellular-signal-regulated kinase (ERK) pathways (Masri et al., 2002; Masri et al., 2004). In addition, the positive cardiac inotropic effects of Apelin are blocked by inhibiting phospholipase C and protein kinase C (Szokodi et al., 2002). Data from the Stainier group showed that stimulating zebrafish Agtrl1b receptor with human Apelin resulted in adenylylcyclase inhibition, which suggests that Gi subunit could be a downstream component of Agtrl1b in zebrafish. Work from the Raz group has demonstrated that the involvement of Gi and PI3K downstream of Cxcr4b to regulate zebrafish PGCs migration (Dumstrei et al., 2004). Similar experiments could be performed in Apelin/Agtrl1b axis. Subcellular events in PGCs could also be investigated when Apelin signaling is altered. In many migratory cells, chemoattractants that are sensed by G-protein coupled receptors signal through phosphatidylinositol-3-OH kinase (PI3K) to recruit pleckstrin homology (PH) domain-containing proteins to the leading edge (Chung et al., 2001; Iijima et al., 2002). Therefore, we could aim to investigate the subcellular localization of a PH–GFP fusion protein (Meili et al., 1999) in PGCs *in vivo*. In wild-type PGCs, membrane recruitment of PH–GFP is locally restricted to the site of lamellipodium protrusion and remains relatively stably positioned over a longer period of time (Knaut et al., 2003). However, such polarized recruitment of PH-GFP in migrating PGCs has not been corroborated by other groups (Raz group studies). Changed chemotaxis in zebrafish PGCs could be examined by this method with excess of deficit Apelin signaling.

From a more general perspective, how GPCR signaling pathways are involved in vertebrate development is still a big open question to us. A comprehensive study including a subset of > 400 endoGPCR members will increase our knowledge in this field.

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