ROLES OF RHO SMALL GTPASE IN ZEBRAFISH DEVELOPMENT

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Table of Contents

Summary

RhoA small GTPase, a member of Ras superfamily, plays pivotal roles in a wide variety of cellular events including cell motility, cell morphology, cell adhesion, differentiation, apoptosis and cell proliferation. It is also important for embryonic development, such as dorsal closure, gastrulation movements, head involution, segmentation and organogenesis. However, majority of these *in vivo* studies of RhoA have been done in the invertebrate model, *Drosophila*, while findings from other animal models may not reflect the specific function of RhoA due to non-specific inhibition of other closely related members of the RhoA family with the use of inhibitor or expression of the dominant negative form of RhoA or Rock. In addition, little is known about the signaling mechanism mediated by RhoA during developmental processes, such as cell movements and cell survival.

To address these questions, *rhoA* gene is cloned from zebrafish, *Danio rerio*, and its temporal and spatial expression profile during embryonic development has been characterized. By capitalizing on the specific functional knockdown using morpholinos against *rhoA* and the availability of convergence and extension (CE) morphants defective in Wnt signaling, we show that *rhoA* morphants are reminiscent to noncanonical *wnt* morphants with serious disruption in CE movements. Injection of *rhoA* mRNA effectively rescues such defects in *wnt5* and *wnt11* morphants. Furthermore, CE defects in *rhoA* or *wnt* morphants can be suppressed by ectopic expression of the two mammalian Rho effectors, Rho kinase (Rock) and Diaphanous (mDia). These results provide the first evidence that RhoA *in vivo* acts downstream of Wnt5 and Wnt11 to regulate CE movements during zebrafish gastrulation without affecting cell fate.

Besides determining the function of RhoA in mediating zebrafish gastrulation movements through regulation of non-canonical Wnt signaling, I also explores the *in vivo* signaling mechanism of RhoA during post-gastrulation period of embryogenesis. Knockdown of RhoA function leads to extensive apoptosis during embryogenesis, resulting in an overall reduction of body size and body length. These defects are associated with reduced activation of growth-promoting Erk and decreased expression of anti-apoptotic *bcl-2*. Moreover, ectopic expression of *rhoA*, *Mek* or *BCL-2* mRNA rescues such phenotypes. Consistently, combined suppression of RhoA and Mek/Erk or Bcl-2 pathways by suboptimal dose of *rhoA* morpholino and pharmacological inhibitors for either Mek (U0126) or Bcl-2 (HA 14-1) can induce developmental abnormalities and enhanced apoptosis, similar to those caused by effective RhoA knockdown. Furthermore, U0126 abrogates the rescue by RhoA and MEK but not BCL-2. In contrast, HA14-1 effectively abolishes all functional rescues by RhoA, MEK or BCL-2, supporting that RhoA prevents apoptosis by activation of Mek/Erk pathway and upregulation of *bcl-2* expression. In addition, both Mek and BCL-2 can rescue gastrulation defects in RhoA morphants. Taken together, these findings reveal an important genetic and functional relationship between RhoA with Mek/Erk and Bcl-2 for cell survival and cell movements control during embryogenesis, and demonstrate the suitability of zebrafish for studying signaling mechanism of various classes of small GTPases in regulating cell dynamics *in vivo*.

List of Tables

List of Figures

List of Abbreviations

ATP — adenosine triphosphate

BCIP — 5-bromo-3-chloro-3-indolyl phosphate

Bp — base pair

BSA — bovine serum albumin

cDNA — DNA complementary to RNA

CE — convergence and extension

CNS — central nervous system

Cyc — cyclops

ddH_2O — double distilled water

DEPC — diethyl pyrocarbonate

DIG — digoxigenin

DMSO — dimethylsulphoxide

DNA — deoxyribonucleic acid

dNTP — deoxyribonucleotide triphosphate

DTT — dithiothreitol

EDTA — ethylene diaminetetraacetic acid

EST — expressed sequence tag

EtOH — ethanol

FCS — fetal calf serum

FGF — fibroblast growth factor

FGFR — fibroblast growth factor receptor

GFP — green flurorescent protein

GTP — guanosine triphosphate

H_2O — water

H_2O_2 — hydrogen peroxide

HCl — hydrochloric acid

HEPES — hydroxyethylpiperazine ethanesulfonate

hpf —hours post fertilization

kb — kilo base pair

KCl — potassium chloride

KH_2PO_4 — potassium dihydrogen phosphate

KOAc — potassium acetate

LB — Luria-Bertani medium

LiCl — lithium chloride

MBT — mid blastula transition

$MgCl_2$ — magnesium chloride

$MgSO_4$ — magnesium sulphate

MMLV — Moloney murine leukemia virus

$MnCl_2$ — manganese chloride

MO — morpholino

mRNA — messenger ribonucleic acid

Na_2HPO_4 — disodium hydrogen phosphate

NaCl — sodium chloride

NaOAc — sodium acetate

NaOH — sodium hydroxide

NBT — nitroblue tetrazolium

NCBI — national centre for biotechnology information

ntl — no-tail

NTP — ribonucleotide triphosphate

PAGE — polyacrylamide gel electrophoresis

PBS — phosphate-buffered saline

PBST — phosphate-buffered saline with 10% tween-20

PCR — polymerase chain reaction

PFA — paraformaldehyde

RACE — rapid amplification of cDNA ends

RNA — ribonucleic acid

rpm — revolution per minute

RT-PCR — reverse transcriptase-polymerase chain reaction

SDS — sodium dodecylsulfate

shh — sonic hedgehog

SRF — serum response factor

SSC — sodium chloride-trisodium citrate solution

SSCT — sodium chloride-trisodium citrate solution with 10% tween-20

TEMED — N,N,N’,N’-tetramethylethylene-diamine

TF— transcription factors

tRNA — transfer ribonucleic acid

UTR — untranslated region

WISH — whole-mount in situ hybridization

YSL — yolk syncytial layer

ZFIN — zebrafish information network

TUNEL — terminal transferase dUTP nick end labeling

List of Publications

Publications relating to research works from the current thesis

1. **Zhu S**, Korzh V, Gong Z, Low BC. (2007) RhoA prevents apoptosis during zebrafish embryogenesis through activation of Mek/Erk pathway. *Oncogene* 27(11):1580-1589.

2. **Zhu S**, Liu L, Korzh V, Gong Z, Low BC. (2006) RhoA acts downstream of Wnt5 and Wnt11 to regulate convergence and extension movements by involving effectors Rho Kinase and Diaphanous: Use of zebrafish as an in vivo model for GTPase signaling. *Cell Signal*.18(3):359-372.

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1. Kong X, Li Z, Gou X, **Zhu S**, Zhang H, Wang X, Zhang J. (2002) A Monomeric L-Aspartase Obtained by in Vitro Selection. *J Biol Chem* 277(27):24289-93.

2. Kong X, **Zhu S**, Gou X, Wang X, Zhang H, Zhang J. (2002) A Circular RNA-DNA Enzyme Obtained by in vitro Selection. *Biochem Biophys Res Commun.* 292(4):1111-5.

3. Kong X, Liu Y, Gou X, **Zhu S**, Zhang H, Wang X, Zhang J. (2001) Directed evolution of α-aspartyl dipeptidase from Salmonelia typhimurium. *Biochem Biophys Res Commun.* 289(1):137-42.

4. **Zhu S**, Wang L. (2001) Advanced study in the relationship between the signal transduction of bFGF/FGFR1 and cardiovascular diseases. *Mol Bio Foreign Med Sci.* 23(4):134-7.

5. **Zhu S**, Wang L. (2001) The construction and expression of FGFR1 immunoadhesion. *US Chinese J Micro Immunol.* 3(4):35-9.

Chapter 1 Introduction

Study of the normal physiology and development of organism is important for the understanding of living activity, prevention or cure of disease, and improvement of the quality of life. For a multi-cellular organism, cell-cell communication and signaling transduction is the pre-requisite and basis for the normal function of single cell and whole organism. As important signaling molecules and key regulators of cytoskeleton organization, Rho (Ras homologous) small GTPases play critical roles in a wide variety of biological and developmental processes, including cell morphogenesis, cell adhesion, cell migration, cytokinesis, gene transcription, cell survival, cell proliferation and organogenesis.

1.1 Rho small guanine nucleotide triphosphatases (GTPases)

Rho small GTPases are members of Ras superfamily [Etienne-Manneville *et al.* 2002; Wennerberg *et al.* 2005]. They share around 35% amino acid identity to Ras. Like Ras, they are approximately 21 kDa monomeric small GTPases and are highly conserved in plants, yeast, fruit flies, round worms and mammals [Etienne-Manneville *et al.* 2002]. In contrast to Ras, Rho proteins have a 13 amino acid insertion in the small GTPase domain, which is the characteristic structure feature to distinguish them from other small GTPases [Valencia *et al.* 1991]. Today, based on sequence homology, structure motif and biological function, 22 mammalian genes encoding at least 25 proteins have been identified and further divided into 5 subfamilies: the RhoA-related subfamily (RhoA, RhoB and RhoC); the Rac1-related subfamily (Rac1, Rac1b, Rac2, Rac3 and RhoG); the Cdc42-related subfamily (Cdc42, brain specific C-terminal splice variant G25K, TC10, TCL, Wrch-1, and Wrch-2/Chp); the Rnd subfamily (Rnd1, Rnd2, and Rnd3/RhoE); and the RhoBTB subfamily. Besides the above, there are three additional Rho GTPases, RhoD, Rif and RhoH/TTF, which do not obviously fall into any of these subgroups. The Miro subfamily, Miro-1 and Miro-2, has recently been included in the Rho family too. However, they have very low homology to the other Rho GTPases and lack the Rho specific insertion in their GTPase domains (Figure 1.1, [Wennerberg *et al.* 2004]).

Figure 1.1 Phylogenetic analyses of the Rho GTPases family and representatives of other Ras GTPases superfamily. A phylogenetic tree of the 22 mammalian Rho family members were generated from a ClustalW multiple sequence alignment. Six subfamilies can be further divided, including RhoA-related, Rac-related, Cdc42-related, RhoBTB, Rnd, and Miro proteins. Adapted from [Wennerberg *et al.* 2004].

1.1.1 RhoA family GTPases

Among all the Rho small GTPases, RhoA is the first Ras homologue to be identified from *Aplysia* in 1985 [Madaule *et al.* 1985]. Few years later, Ridley and Hall reported that overexpression of activated RhoA in fibroblasts can induce rapid formation of stress fibers (boundless of actin filaments) and assembly of focal adhesion (sites of cell/matrix contact) [Ridley *et al.* 1992]. This finding strongly impacts on the cytoskeleton field, because it was the first time to address the molecular mechanism underlying the assembly of the two prominent cytoskeleton structures, stress fibers and focal adhesion. On the contrary, no significant attention has been paid to the other two members of RhoA family GTPases, RhoB and RhoC, although they were characterized at the same time as RhoA [Madaule *et al.* 1985]. This is largely due to the findings that overexpression of activated RhoB or RhoC can induce the formation of stress fibers similar as that of RhoA. Besides, the three Rho isoforms share around 85% amino acid sequence identity across their full-length sequence (Figure 1.2, [Wheeler *et al.* 2004]), with highly conserved region at their N-terminal half and relatively divergent sequence close to the C-terminus. The majority of residues important for GTP binding and hydrolysis, and two consensus sequences, named switch I and switch II, involved in the conformational change between the GTP-bound and GDP-bound states, are located at the conserved N-terminal [Bishop *et al.* 2000]. Thus, RhoA family GTPases are thought to be regulated similarly and their functions are redundant. As such, they are often referred to collectively as “Rho”, and no distinction has been made in most experiments. However, several studies have shown that the divergent sequence at the C-terminal of Rho could be targeted by different proteins, resulting in their distinct sub-cellular localization and

variant biological activities [Wang *et al.* 2003]. Hence, these findings redraw attentions to explore the precise biological function of different Rho isoforms and their specific regulation in physiological and pathological processes.

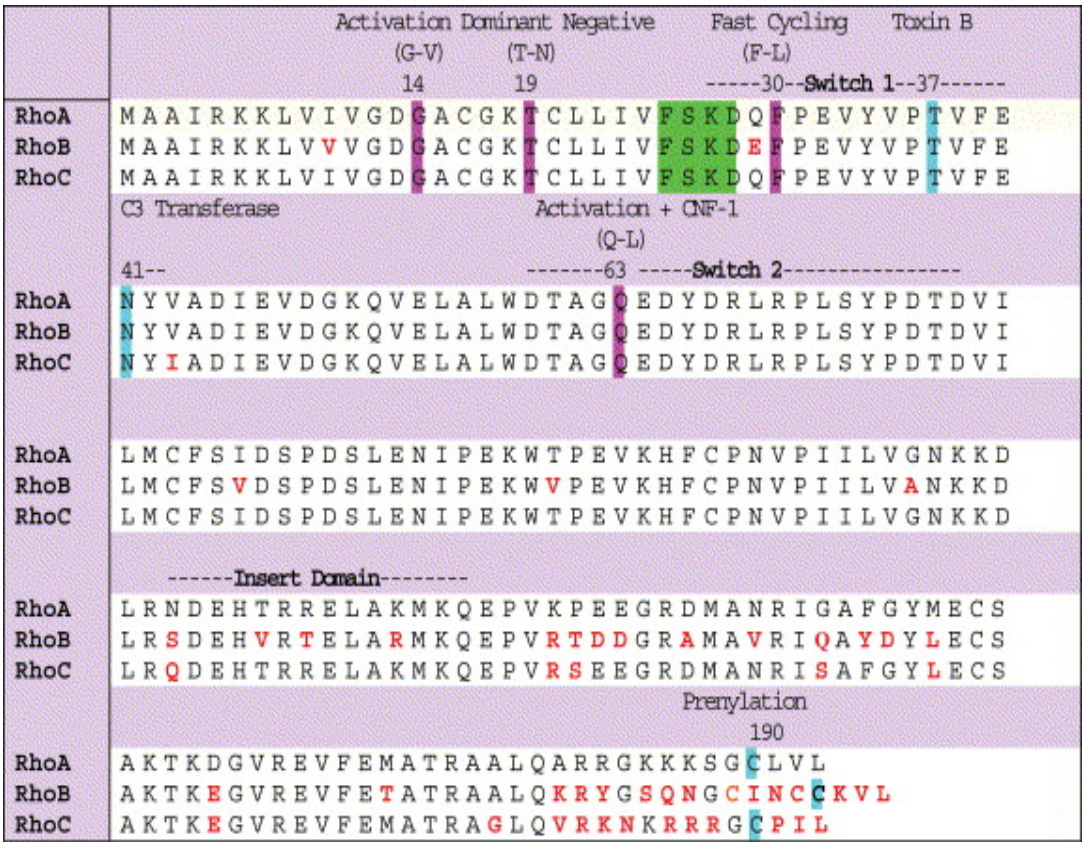

Figure 1.2 Amino acid sequences alignment of mammalian RhoA, RhoB, and RhoC. The divergent residues among RhoA, RhoB and RhoC are indicated in red. The residues that can affect GTPase function by their alteration are indicated in pink. The residues that are targets for toxins are indicated in cyan. The residues that are important for the interaction of Rho with their effectors are indicated in green. The cysteine 4 amino acids at the C terminus, which are critical for the prenylation, are indicated in cyan. Adapted from [Wheeler *et al.* 2004].

1.1.2 Regulation of RhoA family GTPases

Similar as other Rho small GTPases, members of RhoA subfamily cycles between GTP-bound active state and GDP-bound inactive state, and this cycling is tightly controlled by three large families of regulators, including nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). In general, RhoGEFs activate Rho by catalyzing the exchange of GDP to GTP, whereas RhoGAPs stimulate the intrinsic of Rho GTPase activity leading to their inactivation, and RhoGDIs sequester the inactive Rho in the cytosol thus preventing them from their interacting with RhoGEFs and RhoGAPs at the plasma membranes [Hall 2005] (Figure 1.3, [Wang *et al.* 2007]). Because the amino acids important for the interaction with RhoGEFs or RhoGAPs are conserved in all three Rho isoforms, no obvious difference have been detected in the relative activity of these regulators on them in most of studies, except RhoGEF, XPLN, affects RhoA and RhoB but not RhoC [Arthur *et al.* 2002].

In addition to the classical regulation by cycling between an inactive GDP-bound form and an active GTP-bound form, the post-translational modification of Rho proteins also appears to be essential for their subcellular localization, stability and function [Stamatakis *et al.* 2002; Wang *et al.* 2003]. All three Rho isoforms contain a C terminal CAAX motif that is a sequence signal for prenylation (farnesylation or geranylgeranylation). For example, RhoB are shown to be prenylated by either a geranylgeranyl (GG) or a farnesyl (F) isoprenoid group, and further modified by palmitoylation at C-terminal, which facilitates its localization mainly on late endosomes and lysosomes [Wherlock *et al.* 2004]. In contrast, RhoA and RhoC are only

geranylgeranylated, and their further modification is through a polybasic domain close to the C-terminus [Adamson *et al.* 1992a], thereby they are found in the cytoplasm or at the plasma membrane [Adamson *et al.* 1992b; Wennerberg *et al.* 2004; Ridley 2006]. In addition, these post-translational modifications also enhance the association of Rho proteins with membranes, which contributes to their activation on membrane by RhoGEFs and subsequently interacts with their effector proteins to elicit downstream responses [Williams 2003; Wennerberg *et al.* 2004; Rossman *et al.* 2005].

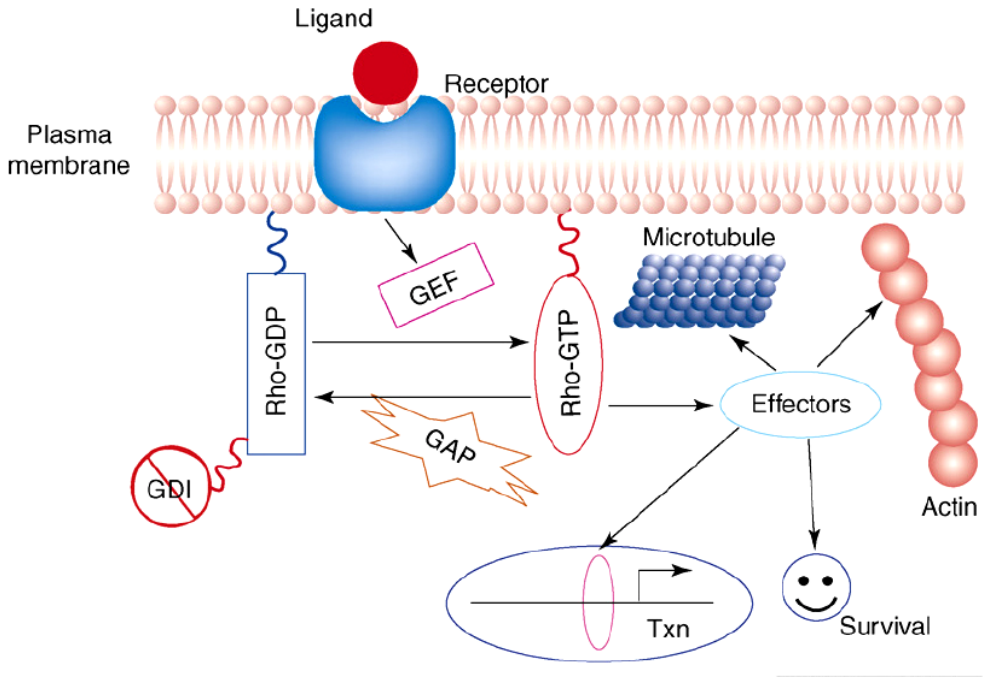

Figure 1.3 The regulation of Rho GTPases. RhoGEFs, RhoGAPs and RhoGDIs are three major families of regulators to control the cycling of Rho GTPase between the active, GTP-bound form and the inactive, GDP-bound form. Upon the stimulation by the extracellular stimuli, RhoGEFs mediate the exchange of GDP to GTP leading to the activation of Rho, while the GTP can be quickly hydrolyzed to GDP by RhoGAPs. Then the GDP bound form of Rho is sequestered in cytoplasm maintaining inactivation by RhoGDIs. Adapted from [Wang *et al.* 2007].

1.1.3 Effectors of RhoA family GTPases

During the past decade, in the attempt to define the biochemical pathways activated by Rho, at least 11 potential targets (downstream effectors) have been identified by means of yeast two-hybrid selection, affinity chromatography techniques or specific interactions with RhoA-GTP bound form [Hall 1998; Kaibuchi *et al.* 1999]. Similar to RhoGEFs and RhoGAPs, the interaction of three Rho isoforms with their effectors is primarily through the conserved switch 1 and 2 regions, implicating that RhoA, RhoB and RhoC share overlapping targets [Wheeler *et al.* 2004]. However, the amino acids sequence in the Rho-binding domain has been found to be different in some of the Rho effecters [Kaibuchi *et al.* 1999], which suggests that the binding ability of effectors to Rho proteins may be variant. In fact, RhoC has been suggested to act more efficiently on Rho kinase and Citron, compared to RhoA and RhoB [Sahai *et al.* 2002b], but the underlying mechanism and the effect on their functions remain largely unknown.

Rho kinase (Rho-associated kinase/ROK/ROCK) is the first kinase effector of RhoA to be discovered. It has been reported to be directly associated with the major activities of RhoA, namely formation of stress fibers and assembly of focal adhesions [Leung *et al.* 1995; Ishizaki *et al.* 1996]. Two ROCKs have been identified, p164ROKα (ROCK2) and p160ROKβ (ROCK1). They contain multiple domains proteins with a highly conserved kinase domain (90% identity) at the N-terminal, a coiled-coil domain in the middle, and a Rho-binding domain together with a pleckstrin homology-like domain at their C-terminal. The C-terminal region is a putative autoinhibitory domain of ROCKs. Upon binding to Rho GTP through the Rho-binding domain, ROCKs adopt open conformations and expose their N-terminal catalytic domains, leading to activation of

downstream signal cascades [Amano *et al.* 2000]. As Ser/Thr protein kinases, ROCKs have been shown to phosphorylate a serial of substrates. Two of them, which are likely to be key regulators for actomyosin assembly, are myosin light chain (MLC) [Amano *et al.* 1996; Kawano *et al.* 1999; Wiedemann *et al.* 2006] and myosin-binding subunit (MBS) of MLC phosphatase [Kimura *et al.* 1996]. ROCKs can increase the levels of phosphorylated MLC by phosphorylating it directly or through the inactivation of the MBS of MLC phosphatase. This leads to enhanced actomyosin assembly and contractility, resulting in the formation of stress fibers and focal adhesions [Maekawa *et al.* 1999; Burridge *et al.* 2004]. Besides, ROCKs can stabilize filamentous actin through activation of LIM-kinase phosphorylation, which subsequently phosphorylates and inactivates cofilin [Maekawa *et al.* 1999]. In addition, other proteins have also been reported to be the substrates of ROCKs, including ERM (ezrin/ radixin/moesin) family of proteins, a Na1/H1 exchange protein (NHE1), and adducing, all of which had been implicated to mediate ROCKs signals to regulate actin cytoskeletal reorganization [Amano *et al.* 2000].

Although ROCKs have been shown to be essential for RhoA induced stress fibers and focal adhesion formation, their activation alone is not sufficient for these processes. More and more studies have implicated the requirement of mammalian homologue of *Drosophila Diaphanous* (mDia) in the proper formation of stress fibers [Watanabe *et al.* 1997; Watanabe *et al.* 1999]. As proteins of formin-homology (FH) family, mDias have been shown to bind to profilin, an actin monomer-binding protein, through their FH domain [Sohn *et al.* 1994]. This interaction allows them to bind to the barbed ends of actin filaments, which antagonizes the binding of capping protein and allows the recruitment of actin monomers to the filament ends, leading to actin polymerization and

F-actin organization into stress fibers [Watanabe *et al.* 1997; Wasserman 1998; Watanabe *et al.* 1999]. Briefly, in RhoA-induced formation of stress fibers and focal adhesion, ROCKs regulate myosin light chain phosphorylation, leading to the bundling of F-actin and clustering of extracellular matrix-ligated integrins, while mDias correct the aberrant orientation of ROCK-induced actin bundles and cooperate with ROCKs for the alignment of these bundles [Watanabe *et al.* 1999]. Besides the above, mDias are also essential for microtubule stabilization at the leading edge of migrating cells [Palazzo *et al.* 2001; Palazzo *et al.* 2004]. As the microtubules and the actin cytoskeleton are coordinately involved in a variety of biological events [Lauffenburger *et al.* 1996; Ishizaki *et al.* 2001], the cooperative regulation of actin cytoskeleton rearrangement and microtubules dynamics by ROCKs and mDias would be very critical for both physiological and pathological processes.

Besides ROCKs and mDias, more and more effector proteins of RhoA have been discovered, including protein kinases (protein kinase N/protein kinase C-related kinase (PKN/ PRK1), PRK2, citron kinase), non-kinases (Rhophilin, Rhotekin, Kinectin), lipid kinase (phospholipase D (PLD), and phosphatidylinositol 4-phosphate 5-kinase (PIP5K)) [Aspenstrom 1999; Bishop *et al.* 2000]. Some of them have been shown to play important roles in the RhoA-mediated actin cytoskeleton reorganization. For example, citron kinase controls actomyosin contraction in RhoA-regulated cytokinesis [Madaule *et al.* 1998], and PIP5K catalyses the formation of phosphatidylinositol 4,5-bisphosphate (PIP2), a regulator of actin-binding proteins [Janmey 1994; Homma *et al.* 1998]. Although the functions of some effectors are still not clear, the diverse targets suggest that Rho

proteins could be involved in a multitude of cytoplasmic signaling cascades to mediate their functions in various biological events.

1.1.4 Functions of RhoA family GTPases

After the role of RhoA in the formation of stress fibers and assembly of focal adhesion was first reported by Ridley and Hall in 1992 [Ridley *et al.* 1992], RhoA family proteins have been brought out from the shadow of Ras family members and to the center stage. In the numerous studies, RhoA has been demonstrated to be a key regulator for cytoskeleton reorganization and involved in a variety of cellular activities including cell migration, cell morphology, cytokinesis, endocytosis and phagocytosis. Besides, RhoA has also been shown to be essential for gene transcription, cell survival, cell cycle progression and cell differentiation [Etienne-Manneville *et al.* 2002].

1.1.4.1 Functions of RhoA in cell biology

1.1.4.1.1 Cell migration

The way in which Rho controls cell movement represents the good regulatory systems involving the actin cytoskeleton rearrangement. For a cell to migrate, one of the prerequisites is the definition of the leading and tailing ends, which is primarily controlled by the spatial distribution of RhoA, Rac, and Cdc42 in cells through reorganization of actin cytoskeleton. In migrating cells, Cdc42 and Rac are often seen at the protruding edge whereas RhoA is seen at the retracting end [Nobes *et al.* 1995]. Generally, activation of Cdc42 at the cell front induces actin polarization and formation of filopodia, defining the leading edge and the direction for cells to migrate [Etienne-Manneville *et al.* 2002; Itoh *et al.* 2002], whereas activated Rac at the cell front

stimulates the dendritic organization of lamellipodia, providing a protrusive force for cell directional migration [Pollard *et al.* 2003]. In contrast, RhoA is primarily activated at the tail of cells, which induces cell body contraction and rear end retraction through promoting focal adhesion assembly and cell contractility [Nobes *et al.* 1999; Kurokawa *et al.* 2005]. In some situations, RhoA can also be activated at the leading edge of migrating cells. For example, high RhoA activity is detected in both cell protruding and retarding ends by fluorescence resonance energy transfer (FRET) biosensors in randomly migrating fibroblasts and epithelial cells, [Pertz *et al.* 2004; Kurokawa *et al.* 2005]. Nevertheless, the spatial-temporal localization of Rho GTPase and the basic mechanism of cytoskeleton rearrangement regulated by them are consistent in plenty of cells [Nobes *et al.* 1999]. In addition, the crosstalk between Rho and Rac can also be controlled spatially. For instance, ROCK is found to suppress cell protrusion in a variety of cells. Recently, it has been shown that inhibiting of Rac induces lamellipodia formation through activation of one of the RacGAPs, FilGAP [Ohta *et al.* 2006]. Therefore, the proper spatiotemporal regulation of Rho GTPases and their functional cooperation is essential for cell migration.

In addition to the determination of the tailing end before the commencement of cell movement, RhoA is primarily involved in two aspects during migration, generating actomyosin-based contractility in the cell body and promoting focal adhesion turnover at the rear of the cell. This is mainly elicited by two of its downstream effectors, ROCK and mDia. As we mentioned before, ROCK can activate LIMK by phosphorylation, which leads to inactivation of cofilin, thereby stabilizing the actin filaments [Maekawa *et al.* 1999; Sumi *et al.* 2001]. ROCK can also phosphorylate MLC and MBS of MLC phosphatase, resulting in increased level of phosphorylated MLC, which in turn leads to

the cross-linking of actin filaments and generation of contractile force, consequently promoting cell movement [Mitchison *et al.* 1996]. MDia, on the other hand, cooperates with ROCK in the assembly of actin myosin filaments [Uehata *et al.* 1997; Watanabe *et al.* 1999] and regulates microtubule (MT) dynamics. It has been reported that activated mDia1 can induce longitudinally aligned MTs parallel to F-actin bundles along the long axis of the cell, and overexpression of GFP-mDia2 has been found to co-localize with Glu-MTs, leading to their stabilization [Ishizaki *et al.* 2001; Palazzo *et al.* 2001]. Also, mDia is able to mediate integrin-FAK signaling to facilitate MT stabilization in the leading edge of migrating cells [Palazzo *et al.* 2004]. Thus, through the cooperative regulation of actin cytoskeleton rearrangement and microtubule stabilization by ROCKs and mDias, RhoA promotes contractility during cell migration.

In addition, focal adhesion turnover mediated by ROCK in the tail of migrating cells is also necessary for cell migration [Rodriguez *et al.* 2003; Small *et al.* 2003]. For instance, ROCK has been reported to be able to increase the number and size of integrin-based focal adhesions in many different types of adherent cells [Ridley 2000; Linder *et al.* 2003], and induces retraction of focal adhesions by strong actomyosin contraction, resulting in detachment of the tail in migrating cells [Meng *et al.* 2004]. Thus inactivation of RhoA leads to the formation of an elongated tail and failure in cell movement [Rodriguez *et al.* 2003; Small *et al.* 2003]. Taken together, through the precise spatiotemporal localization and specific activation of target proteins, RhoA cooperatively controls contractility and focal adhesion turnover during cell migration.

1.1.4.1.2 Cell morphology

Similar to the regulation of cell movement, the basic mechanism of cytoskeleton rearrangement in the assembly of focal adhesion and generation of contractility mediated by RhoA is rather consistent with the establishment of cell morphology. For instance, RhoA has been shown to be essential for cell-cell adhesion, particularly for adherent junctions (AJs) and tight junctions (TJs) which are the major intercellular adhesive junctions for the establishment of epithelial cell shape. The AJs provide a strong mechanical connection between adjacent cells, whereas TJs form a physical barrier preventing the diffusion of both proteins and lipids between the apical and basolateral membranes. Studies show that the inhibition of endogenous RhoA by the bacterial toxin C3 transferase can inhibit the formation of both AJs and TJs, through disrupting the organization of actin filaments [Narumiya *et al.* 1993; Braga *et al.* 1997; Takaishi *et al.* 1997; Zhong *et al.* 1997]. Similarly, overexpression of dominant negative mutant of ROCKs or Dias disrupts cytoskeletal organization, which leads to the partial perturbance or removal of cadherin receptors from newly formed or mature junctions, resulting in a loss of tension at these junctions [Nusrat *et al.* 1995; Jou *et al.* 1998]. In addition, the contractile event mediated by RhoA is also involved in the regulation of cell morphology. In macrophage cells and neuronal cells, activation of RhoA leads to cell rounding, which is resulted from the formation of contractile actin-myosin filaments, but not focal adhesions. In contrast, the flattened shape of fibroblasts is not affected by RhoA activation, possibly due to the formation of strong focal adhesion [Aepfelbacher *et al.* 1996; Katoh *et al.* 1996; Postma *et al.* 1996; Tigyi *et al.* 1996; Allen *et al.* 1997; Kozma *et al.* 1997]. Hence, the differential regulation of cell adhesion and cell contractility by RhoA could lead to different changes in cell morphology.

1.1.4.1.3 Cytokinesis

Cytokinesis, as the final step towards cell division, also requires Rho GTPases-dependent spatial and temporal control of actin and microtubules. The direct evidence of the involvement of RhoA in cytokinesis lies in its restricted activation in the cortex prior to and during furrowing, which is revealed by the expression of fusion protein Rhotekin-GFP in echinoderm or vertebrate embryonic cells. Bement and co-workers report that active RhoA during anaphase is accumulated at a restricted zone and the width of the zone remains constant during cleavage [Bement *et al.* 2005]. Besides, authors notice that RhoA may function in different stages during cytokinesis including centrosome separation, spindle orientation, chromosome congression, and contractile ring formation [Bakal *et al.* 2005]. For instance, knockdown of *rho1* in *C. elegans* affects the cortical dynamics and centrosome positioning [Sonnichsen *et al.* 2005]. Similarly, inhibition of ROCK in cells by pharmacological inhibitor, Y-27632, impairs centrosome separation and results in aberrant spindle phenotypes [Rosenblatt *et al.* 2004].

In comparison with centrosome separation and spindle orientation during cytokinesis, the role of RhoA in contractile ring formation is more extensively studied. Three of RhoA downstream targets, ROCK, mDia, and Citron kinase (Citron K), have been demonstrated to be essential for this process. Inhibition of anyone of them prevents the assembly of the contractile ring in a variety of mammalian cells resulting in multinucleate cells [Bhattacharyya *et al.* 2003]. Briefly, mDia1 localizes to the cleavage furrow during cytokinesis [Wallar *et al.* 2003], where it promotes local actin polymerization and/or coordinates microtubules with actin filaments at the site of the contractile ring. ROCK stimulates actomyosin assembly to generate the contractile force

that is necessary for driving contractile ring ingression, while Citron K, localizes to the cleavage furrow in a RhoA-dependent manner, and seems to be necessary for completion of cytokinesis by stably maintaining the ring components anillin and actin at the midbody [Eda *et al.* 2001; Shandala *et al.* 2004]. Besides, Rho upstream regulators, RhoGEFs, also contribute to cytokinesis through regulation of RhoA activity [Bement *et al.* 2005; Glotzer 2005]. It has been reported that the *Drosophila* RhoGEF, *Pebble*, can interact with components of centralspindlin complexes to mediate formation of the contractile ring [Somers *et al.* 2003]. Similarly, knockdown of RhoGEF, *ECT2*, using RNAi leads to complete inactivation of RhoA at the restricted zone where the contractile ring will be formed, and prevents localization of both actin and myosin II in the contractile ring and ingression [Yuce *et al.* 2005; Zhao *et al.* 2005].

Taken together, all the above studies show the crucial functions of RhoA in cell biology through its regulation of actin and microtubule dynamics. In addition to the direct effect on cytoskeleton, RhoA also plays a role in gene expression, which makes it more important in a wider variety of biological events, such as cell proliferation and cell survival control.

1.1.4.1.4 Cell proliferation

The early implication of RhoA in cell proliferation comes from the observation that its inactivation can inhibit mitogen-stimulated G1–S phase progression in Swiss 3T3 fibroblasts, whereas its activation triggers progression of the G1 phase in quiescent fibroblasts [Yamamoto *et al.* 1993; Olson *et al.* 1995]. Further studies show that RhoA could regulate G1-S phase progression via at least two ways: activation of cyclin D1

transcription and inhibition of cyclin/Cdk (cyclin-dependent kinase) expression [Olson *et al.* 1995; Olson *et al.* 1998; Welsh *et al.* 2001]. For instance, RhoA has been reported to sustain the activation of extracellular-signal-regulated kinase (ERK) under the stimulation of fibroblast-growth-factor, which is essential for cyclin D1 expression [Hirai *et al.* 1997; Laufs *et al.* 1999]. It can also function as the master of adhesion-dependent regulator of cyclin D1 expression, through its control in the assembly of integrins complex [Assoian *et al.* 1997; Welsh *et al.* 2001]. On the other hand, activation of RhoA has been shown to suppress Cdk inhibitor p21 and p27 transcription [Auer *et al.* 1998; Hu *et al.* 1998; Olson *et al.* 1998], whereas inactivation of RhoA leads to increased expression of these genes [Weber *et al.* 1997; Rivard *et al.* 1999]. Similarly, inhibition of RhoA signaling in vascular smooth muscle cells upregulates p27Kip1 expression and inhibits cell proliferation [Laufs *et al.* 1999], suggesting modulation of the expression of Cdk inhibitors by RhoA is important for cell proliferation.

1.1.4.1.5 Cell survival

To date, the mechanism underlying RhoA-mediated cell survival is largely unknown, but several anti-apoptotic pathways have been implicated in the RhoA-dependent suppression of apoptosis. For example, the expression of constitutively-active RhoA induces assembly of cortical F-actin to promote activation of ERK and facilitates glomerular epithelial cells survival [Bijian *et al.* 2005], while over-expression of Rho downstream effector, Rhotekin, in human gastric cancer confers cell resistance to apoptosis through activation of NF-kB pathway [Liu *et al.* 2004]. Inhibition of RhoA can activate caspase-9- and caspase-3-dependent apoptosis in human umbilical cord vein endothelial cells [Hippenstiel *et al.* 2002] and induce p53 or other proapoptotic proteins

in human endothelial cells [Li *et al.* 2002]. Besides, RhoA has been reported to induce anti-apoptotic Bcl-2 expression in various cell lines, including murine T cell line [Gomez *et al.* 1997], vascular smooth muscle cells [Blanco-Colio *et al.* 2002], and human osteosarcoma cells [Fromigue *et al.* 2006].

However, reports on the role of RhoA in cell survival are rather contradictory. Overexpression of RhoA is found to induce apoptosis in a range of cell lines, including NIH3T3 fibroblasts, human erythroleukemia K562 cells, and erythroblast cell lines. The RhoA-induced apoptosis appears to be associated with enhanced ceramide level or reduced Bcl-2 expression [Jimenez *et al.* 1995; Esteve *et al.* 1998]. Besides, the dynamic rearrangement of actin cytoskeleton by ROCK has been shown to be involved in the morphological changes in cell apoptosis. For instance, ROCK I can be cleaved by caspase 3 directly, leading to its activation, which subsequently generates actin-myosin contractile force and results in cell contraction and membrane blebbing [Leverrier *et al.* 2001; Sebbagh *et al.* 2001] in apoptotic cells. In addition, catalytical activation of PRK1/PKN, another downstream effector of RhoA, has also been reported to induce apoptosis by promotion of actin stress fiber disassembly [Coleman *et al.* 2002]. However, several other studies suggest that the activation of RhoA might not be responsible for the apoptotic contraction and blebbing in the initiation of cell apoptosis. For example, pro-apoptotic stimulus can not activate RhoA in Swiss 3T3 or NIH 3T3 cells [Coleman *et al.* 2001] and inactivation of RhoA by bacterial toxin C3 transferase did not inhibit membrane blebbing in apoptotic cells [Coleman *et al.* 2001; Sebbagh *et al.* 2001]. Therefore, how RhoA signaling contributes to cell survival needs to be further elucidated.

1.1.4.2 Functions of RhoA in animal development

1.1.4.2.1 Embryonic morphogenesis

During embryonic development, cells undergo a range of morphological changes in their shape, polarity and cell-cell contact to form well-organized tissues, organs and whole embryos. Thus, RhoA, as a key regulator for the organization of actin cytoskeleton, has been shown to be necessary for these morphogenic processes. In *Drosophila*, loss of *RhoA* results in abnormal epithelial cells shape, disorganization of cells along the dorsal midline or in the internalization of anterior head structures, which consequently leads to an opening in dorsal closure and head [Lu *et al.* 1999b; Magie *et al.* 1999]. Overexpression of *Rho1* specifically in *Drosophila* eyes can induce severe rough eye defects with a grossly abnormal morphology of the rhabdomeres [Hariharan *et al.* 1995]. Consistently, in *Xenopus*, overexpression of *XRhoA* increases cell adhesion by antagonizing *XRnd1*, which in turn affects head formation [Wunnenberg-Stapleton *et al.* 1999].

In addition to the control of actin dynamics, RhoA signaling could contribute to morphogenesis by regulation of cell size. In S. cerevisiae, the daughter cells of *rhoA* mutant or its downstream effectors mutants (*skn7p*, *fks1p* and *mpk1p*) display abnormally small size. This is probably due to the failure of mother cells (carrying mutation in *rhoA* or its effectors) in reaching an appropriate size before budding, or premature entry to mitosis [Kikuchi *et al.* 2007]. Besides, RhoA can regulate cell size by modulating IGF-induced phosphorylation of cAMP response element binding protein (CREB). In p190-B RhoGAP knockout mice, abnormally high levels of active Rho protein were suggested to be associated with defects in CREB activation upon exposure to insulin or IGF-1, thereby

leading to 30% reduction of embryo size [Sordella *et al.* 2002]. Taken together, these studies suggest that RhoA is involved in different aspects in embryonic morphogenesis including control of cell shape, cell adhesion, and cell size.

1.1.4.2.2 Cell movement

In addition to the dramatic cell morphological changes, the accurate migration of cells to proper sites in response to specific cues is also critical for normal embryonic development. In *Drosophila*, one of the most severe defects in *Rho1* loss of function mutants is imperfect dorsal closure. During normal *Drosophila* dorsal closure, the lateral epithelial sheets migrate towards dorsal midline and cover the dorsal region of the embryo. In contrast, this process is disrupted in *Rho1* mutant and cells are disorganized at dorsal midline, which results in a big hole or "dorsal open" on the dorsal surface of embryos [Magie *et al.* 1999]. Similar defects are also observed in the loss of function mutant of *Drosophila* PKC-related protein kinases (*Pkn*), suggesting that *Pkn* could be one of the downstream effectors mdeiating *RhoA*-dependant cell movement during dorsal closure [Lu *et al.* 1999a]. Another characteristic zygotic defect of *Rho1* mutant is in head involution, which is the result from the failure in internalization of anterior head structures [Magie *et al.* 1999]. Consistently, RhoA is also required for the invagination of epithelial cell and transepithelial migration of germ cell during *Drosophila* embryogenesis [Simoes *et al.* 2006]. Similarly, in *C. elegans*, disruption of cell movements, such as epidermal P-cell migration, is also observed when *rhoA* signaling is inhibited either by the expression of dominant negative mutant of *rho-1* or knockdown of *ect-2*, a GEF for RhoA [Spencer *et al.* 2001; Morita *et al.* 2005]. In *Xenopus*, *RhoA* has been shown to mediate p120 catenin signaling in the control of the migration of cranial

neural crest cells from the neural tube into the branchial arches [Ciesiolka *et al.* 2004]. Besides, RhoA also plays a critical role in gastrulation movements and midline convergence of organ primordia during *Xenopus* and zebrafish embryogenesis, which will be reviewed in detail in the section *1.2.2.2.2*. Taken together, RhoA is critical for proper movements of multiple types of cells in different developmental stages during both invertebrates and vertebrates embryogenesis.

1.1.4.2.3 Cell growth and survival

In addition to the cell movement control, RhoA also plays pivotal roles in cell growth and cell survival during animal development. It has been shown that inhibition of RhoA activity by C3 transferase in murine thymus leads to a decrease in the number of thymocytes by increasing apoptosis and reducing proliferation [Henning *et al.* 1997]. Similarly, cardiac–specific inhibition of Rho by overexpression of RhoGDI in transgenic mice or inhibition of ROCK in cultured murine embryos disrupts cardiac morphogenesis and inhibits cardiomyocyte proliferation, but cell survival is not affected in both cases [Wei *et al.* 2002; Zhao *et al.* 2003]. Another study reveals that suppression of RhoA-ROCK signaling by conditional expression of dominant negative RhoA or ROCK in transgenic mice reduces the number of motor neurons in the spinal cord by increasing apoptosis [Kobayashi *et al.* 2004]. Thus, the proper expression of RhoA is necessary for both cell proliferation and cell survival during organogenesis, but whether this also affects early embryonic development is still unclear.

1.1.4.3 Other functions of RhoA family GTPases

In addition to the above developmental processes, more and more important roles of RhoA in animal development have been explored. This includes oogenesis, segmentation, tissue polarity [Strutt *et al.* 1997], T cell development [Corre *et al.* 2001], smooth muscle contraction [Webb 2003], skeletal muscle development [Charrasse *et al.* 2003], cardiac morphogenesis [Wei *et al.* 2002; Kaarbo *et al.* 2003], head formation [Wunnenberg-Stapleton *et al.* 1999], and so on. Besides, RhoA is also involved in various aspects of neural development [Luo 2000], such as proliferation of neuroblasts [Lee *et al.* 2000], growth cone collapse, axon guidance [Bito *et al.* 2000], neurite outgrowth, dendritic patterning [Nakayama *et al.* 2000a; Pilpel *et al.* 2004], spine morphology [Nakayama *et al.* 2000b; Tashiro *et al.* 2000], and neurotransmitter release [Bito *et al.* 2000; Lee *et al.* 2000; Nakayama *et al.* 2000b; Tashiro *et al.* 2000; Huot 2004; Pilpel *et al.* 2004].

Although the biological functions of RhoA have been studied in various *in vitro* and *in vivo* systems, many of the findings on its functions are obtained using expression of its constitutive active or dominant negative mutants, thus neglecting the possibility of functional interference by non-specific inhibition or activation of other closely related members. As we mentioned earlier, the primary protein sequence of RhoA, RhoB and RhoC are around 85% identical, and the majority of amino acids and motifs important for their interaction with regulators and effectors are rather conserved, which results in their extensive overlapping of RhoGEFs, RhoGAPs and target proteins [Wheeler *et al.* 2004]. As such, the overexpression of dominant-negative (DN) form of RhoA (T19N) may inhibit all three endogenous Rho signaling by sequestering their common upstream RhoGEFs, whereas the ectopic expression of constitutively activate (CA) mutant of

RhoA (G14V) may activate multiple downstream target proteins without distinction, leading to unspecific activation of all three Rho signaling [Wang *et al.* 2007]. Hence, found that overexpression of DNRhoA (T19N) can also inhibit RhoC-induced cancer cell migration and invasion [Clark *et al.* 2000], and more and more distinct function of individual Rho isoforms has been uncovered. For example, RhoB can act opposite to RhoA and RhoC on cell proliferation [Du *et al.* 1999; Chen *et al.* 2000b], and RhoB, but not RhoA and RhoC, can mediate the transport of late endosomes [Mellor *et al.* 1998; Gampel *et al.* 1999]. Consistently, in the *in vivo* studies, knockout RhoA leads to early embryonic lethality, while knock out of RhoB or RhoC do not cause obvious embryonic defects [Wang *et al.* 2007]. Therefore, application of proper approaches that could specific target to individual Rho proteins would help us better understand the precise gene functions and signaling capacity of Rho isoforms in both cellular and developmental processes.

1.1.5 Functions of RhoA family GTPases in pathophysiological processes

Being such an important player for proper cell behavior and normal development, the deregulation of Rho signaling pathway has been correlated with many pathophysiological processes, including tumorgenesis and progression, vascular disease, neural degeneration diseases, and so on.

1.1.5.1 Tumorgenesis, invasion and metastasis

The implication of RhoA in tumorgenesis comes from its high level of activity in a variety of cancers including colon, breast, lung, testicular germ cell, head, and liver [Sahai *et al.* 2002a] and its close family members, RhoB and RhoC, are also implicated to

be cancer-promoting genes [Wheeler *et al.* 2004]. It has been suggested that the involvement of RhoA in tumorgenesis could be linked to its role in the promotion of cell cycle progression. For example, RhoA controls the timing of cyclin D1 induction or induces its expression through sustained activation of ERK [Welsh *et al.* 2001]. RhoA can also inhibit activities of p21Cip1, p27Kip and p16Ink4, important regulators in G1 to S transition [Adnane *et al.* 1998; Olson *et al.* 1998; Laufs *et al.* 1999; Seasholtz *et al.* 2001; Liberto *et al.* 2002; Vidal *et al.* 2002]. In addition to the involvement in tumorgenesis, RhoA contributes to invasion and metastasis [Ridley 2004; Titus *et al.* 2005]. It has been reported that activation of RhoA not only enhances hepatoma cell motility but also promotes cells invasion [Yoshioka *et al.* 1998; Genda *et al.* 1999], while abrogation of ROCK signaling could inhibit cells metastatic potentials and decrease their invasive activities in a variety of tumor cell lines, such as rat MM1 hepatoma cell lines, pancreatic cancer cell-lines, and breast carcinoma cells [Bourguignon *et al.* 1999; Genda *et al.* 1999; Itoh *et al.* 1999; Somlyo *et al.* 2000; Takamura *et al.* 2001; Jo *et al.* 2002]. Thus, RhoA signaling is involved in different stages of tumor development through coordinative regulation of gene transcription and actin cytoskeleton dynamics. Besides, increasing studies have shown that inhibition of RhoA prenylation through various reductases including statins and its derivatives, fluvastatin or lovastatin, can reverse RhoA-dependent tumorgenesis and metastasis [Kusama *et al.* 2002], suggesting the RhoA/ROCK signaling pathway could be the potential target for cancer therapy in future.

1.1.5.2 Cardiovascular disorders

In addition to tumorgenesis and progression, RhoA appears to be involved in a variety of cardiovascular diseases, including atherosclerosis [Mallat *et al.* 2003], glomerulosclerosis [Nishikimi *et al.* 2004], vasospastic angina [Katsumata *et al.* 1997; Sato *et al.* 2000], ischemic stroke [Toshima *et al.* 2000], hypertension [Uehata *et al.* 1997; Higashi *et al.* 2003; Abe *et al.* 2004], myocardial ischemia-reperfusion injury [Bao *et al.* 2004; Wolfrum *et al.* 2004], neointima formation [Sawada *et al.* 2000; Matsumoto *et al.* 2004], vascular remodeling [Miyata *et al.* 2000], and endothelia dysfunction [Laufs *et al.* 1998; Eto *et al.* 2001]. Growing evidence shows that the activity of RhoA/ROCK is often elevated in animal models of hypertension and disorders of the cardiovascular system. Inhibition of ROCK can normalize the arterial pressure in these hypertension models [Uehata *et al.* 1997] and suppress the development of atherosclerosis and arterial remodeling after vascular injury [Sawada *et al.* 2000; Mallat *et al.* 2003]. In addition, accumulating studies implicate that RhoA/ROCK may contribute to cardiovascular diseases through controlling vascular smooth muscle cell proliferation and contraction. For instance, RhoA can induce smooth muscle cell proliferation in response to different stimuli, including angiotensin II, and PDGF [Hengst *et al.* 1996; Laufs *et al.* 1999; Seasholtz *et al.* 1999; Yamakawa *et al.* 2000; Sauzeau *et al.* 2001], and the induced cell proliferation seems associated with deregulation of p27Kip1 activity [Hengst *et al.* 1996; Laufs *et al.* 1999]. Activation of Rho/ROCK pathway can also facilitate and prolong contraction of smooth muscle, which may lead to arterial remodeling during vascular pathologies [Chitaley *et al.* 2001]. In addition, a number of vascular diseases appear to benefit from inhibition of ROCKs by fasudil or statins [Laufs *et al.* 1998; Laufs *et al.* 2000; Takemoto *et al.* 2002; Wolfrum *et al.* 2004], including systemic hypertension

[Masumoto *et al.* 2001], pulmonary hypertension [Fukumoto *et al.* 2005], vasospastic angina [Masumoto *et al.* 2002], stable effort angina [Shimokawa *et al.* 2002], stroke [Shibuya *et al.* 2005], and chronic heart failure [Kishi *et al.* 2005]. Hence, precise and tight regulation of Rho signaling pathway would be promising therapeutics for cardiovascular diseases.

1.1.5.3 Other pathophysical processes

In addition to the most popular and the worst prognostic diseases, cardiovascular disorder or tumor, deregulation of RhoA has been shown to be involved in other diseases, such as Alzheimer disease [Mueller *et al.* 2005], X-linked mental retardation (MRX) [Chelly *et al.* 2001; Frints *et al.* 2002; Negishi *et al.* 2002; Ramakers 2002; Chechlacz *et al.* 2003; Govek *et al.* 2005], bronchial asthma [Chiba *et al.* 2004], demyelinating diseases [Mueller *et al.* 2005], glaucoma [Honjo *et al.* 2001], and osteoporosis [Ohnaka *et al.* 2001]. In order to reveal the molecular mechanism and develop effective therapies for human diseases, understanding of the molecular basis mediated by RhoA in normal physiological processes is important and urgent. Moreover, choosing a proper *in vivo* model system would be necessary and valuable for us to address these issues accurately and effectively.

1.2 Zebrafish model

1.2.1 Zebrafish as an *in vivo* model

Zebrafish, (*Danio rerio*), a small tropical freshwater teleost fish, has been introduced as model system few decades ago. With a combination of various features, it

has emerged as one of the most promising model organisms in the study of developmental biology. Generally, zebrafish is a small size fresh water fish (average 3.8 cm in length for adult fish), thus large numbers of fish can be easily maintained in aquaria labs. It has a short generation time (around 3-4 month to mature) and produces large number of eggs per spawning, which makes it well-suited for genetic analysis. In addition, its embryos are optically transparent, and embryonic development occurs externally and rapidly. Thus, people can easily visualize the whole embryogenesis within a few days. It is also possible to monitor cell movement *in vivo* or internal organogenesis by labeling particular cell types or generation of transgenic lines with fluorescent proteins. Besides, the laboratory methods for its husbandry, and detailed description of developmental staging series during its early embryogenesis have been well established making it one of the most excellent vertebrate models for developmental study [Westerfield 2000].

In addition to the above frequently mentioned advantages of zebrafish, the amenability to various cellular, molecular and genetic techniques makes it a powerful model not only for developmental but also for genetic study. Compared with *Xenopus* embryos, zebrafish embryos are very amenable to microinjection, which makes reverse genetics studies, including loss and gain-of-function studies, relatively easier. Although the techniques of recombinant gene knockout have not been established yet in zebrafish, morpholino (MO) antisense oligo-mediated functional knockdown has provided some significant advantages over knockout mouse models. For instance, by targeting the sequence between 5' UTR and the first 25 bases of coding sequence, translation initiation of the targeted gene can be blocked by MOs [Summerton *et al.* 1997]. As such, the

degree of a given gene knockdown can be controlled by the injection of different doses of MOs, making it possible to study the function of genes that could cause embryonic lethality by complete gene knockout. Moreover, several MOs targeting to different gene products can be co-injected into wild type or mutant embryos for combinational loss of function experiments [Ekker *et al.* 2001]. Additionally, MOs can also alter pre-mRNA splicing by targeting splice junctions in the nucleus, and this technique has been applied successfully to address the function of particular domains *in vivo* by exon(s) deletion or intron(s) retention [Morcos 2007; Summerton 2007]. Besides, the effectiveness and specificity of gene knockdown by MO can be simply confirmed through co-injection of MOs with related DNAs or mRNAs, which is called rescue experiment. In addition to functional knockdown by microinjection of MOs, application of effective pharmacological inhibitors and availability of numerous mutant lines make zebrafish a wonderful model to study the signaling mechanism underlying developmental processes and understand networks within different signaling pathways *in vivo*.

In addition to amenability for loss of function studies, zebrafish is also powerful in gain of function studies. Similar to the rescue experiment, microinjection of DNA or synthetic mRNAs of interest into one-cell stage embryos has been extensively used in zebrafish as a form of gain-of-function study. Besides this early ubiquitous overexpression of gene of interest, cDNAs can also be overexpressed spatially, stably, or inducible under control of different promoters. For instance, fusion of green fluorescent protein (GFP) or red fluorescent protein (RFP) together with genes of interest have been ligated downstream of different tissue-specific promoters, enabling the identification of gene function specifically in tissue or organ development [Gong *et al.* 2001; Zhu *et al.*

2004]. Moreover, under the control of a heat shock-sensitive promoter or tetracycline response element, transgenic gene expression can be induced at any desired stage of development by increasing the water temperature (from 28 to 39 °C) or incubation in water containing tetracycline [Pyati *et al.* 2007]. Similarly, Gal4-UAS and Cre-LoxP systems have also been extensively applied to induce transgenes expression simply by crossing two individual transgenic lines [Scheer *et al.* 1999; Pan *et al.* 2005; Le *et al.* 2007]. Taken together, amenability to various techniques makes zebrafish a promising vertebrate model to discover novel gene functions in development.

Zebrafish has also emerged as an important disease model to unravel the molecular mechanism and therapeutic targets of disease processes. Most of the critical signaling pathways have been shown to be conserved between zebrafish and human, and zebrafish has very similar histology to human. Hence, various diseases, such as tumorgenesis, kidney discord, vascular diseases, Parkinson's disease, have already been studied on zebrafish. For example, in the study of tumor formation, a zebrafish transgenic line in which oncogenic c-Myc can be specifically overexpressed in T-lymphocytes was generated. In this transgenic fish, massive T-cell over-proliferation and metastasis occurred, which makes it a valuable animal model for human T-cell leukemia study [Langenau *et al.* 2003]. Recently, another conditionally inducible human constitutively activated K-ras transgenic line has been generated, which further establishes an *in vivo* tumor model for better understanding of general molecular basis underlying tumorgenesis and malignancy [Le *et al.* 2007]. Besides, zebrafish could be a wonderful model for pharmacological compounds screening, as large number of embryos or larvae can be screened and analyzed at once using very little material [Yeh *et al.* 2003; Zon *et al.* 2005].

Through this high-throughput screening, novel and potential anticancer agents would be discovered without any bias. Moreover, some of these studies have shown promising results in rescuing abnormal vascular network formation [Peterson *et al.* 2004] or cell cycle defects [Stern *et al.* 2005]. Therefore, combined with the power of zebrafish genetics, ease manipulation of gene expression, and elegancy in chemical compound screening, zebrafish is a good model system for exploring the molecular mechanism underlying both normal physiological and pathophysiological processes, and examining the efficacy of potential therapies.

1.2.2 Zebrafish development

1.2.2.1 Stages of embryonic development of zebrafish

The development of zebrafish can be broadly divided into 4 stages, embryo (0 - 3 dpf), larval (4 - 29 dpf), juvenile (30 - 89 dpf) and adult (90 dpf - 2 y). During the embryogenesis (first 3 days after fertilization), five periods can be further defined according to the morphological features, including the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods [Kimmel *et al.* 1995]. After fertilization, embryos enter the first zygotic cell cycle, which is defined as the zygote period (0-0.75 hpf). Following the zygote period is the cleavage period (0.75- 2.25 hpf), in which seven cycles of rapid and synchronous cell division occur. During blastula period (2.25 -5.25 hpf), embryo continues two more synchronous cycles of cell division followed by lengthened and asynchronous cell division together with the commencement of epiboly movement. During gastrula period (5.25 – 10.33 hpf), three more types of movements (involution, convergence and extension) together with epiboly are taking place to shape

the embryonic axis. At the end of gastrulation, three germ layers are formed, and primary organogenesis begins. During segmentation (10.33 – 24 hpf), somites, neuromeres, and pharyngeal arch primordia develop and the tail appears. In the pharyngula period (24-48 hpf), circulation, pigmentation, and fins begin to develop, and embryonic body axis is straightened. Finally, at the hatching period (48-72 hpf), rapid primary organ morphogenesis is completed and embryos hatch out from the chorine.

1.2.2.2 Gastrulation

During the early embryonic development, gastrulation is one of the most fundamental and important developmental processes, during which the basic vertebrate body plan of the zebrafish embryo is established, including the formation of the anterior-posterior and dorsal-ventral axes, and the development of three germ layers — ectoderm, mesoderm and endoderm. Ectoderm is located on the outside of the three-germ layer-embryo, giving rise to the epidermis and neural tissues. The endoderm is on the inside of embryo, which will form the digestive tube and its accessory organs; while the mesoderm between the ectoderm and endoderm is included in the generation of future middle layer of the adult body plan, including muscles, cardiovascular, urogenital and skeletal elements of the body [Kimmel *et al.* 1990].

1.2.2.2.1 Cell movements during gastrulation

During vertebrate gastrulation, four evolutionarily conserved morphogenetic movements have been characterized based on the morphogenetic changes they produce,

namely, epiboly, internalization, convergence and extension [Warga *et al.* 1990]. Epiboly movement starts before the germ layers arise, and continues throughout the gastrulation. In *Xenopus* and zebrafish, radial cell intercalation is the key cell behavior during epiboly, which is characterized by the interdigitation of deeper-layer-cells between more superficial cells or *vice versa*, resulting in the occupation of fewer layers of cells in a big area. Moreover, together with directed cell migration, the epiboly leads to thinning and expansion of surface tissue during gastrulation [Keller 2005] (Figure 1.4, [Montero *et al.* 2004]).

In contrast to epiboly, internalization of mesendodermal precursors is believed to be the defining event of gastrulation. Upon the mesoderm and endodermal precursors moving through the blastopore, internalization, which drives the cells moving from the blastula surface beneath the ectodermal via blastopore, occurs. The pattern of cell movements after invagination is different among vertebrates. For example, in *Xenopus* invagination is followed by involution, in which the cells move as one cohesive sheet [Trinkaus 1996]; whereas in chick and mouse [Kane *et al.* 2002], ingression movement occurs, whereby the mesoderm and endodermal precursors move individually via blastopore. Interestingly, in fish, both movements are involved. The mesendodermal precursors approach the blastopore as a coherent sheet and then loss the coherence, which in turn leads to individual but coordinative cell movements [D'Amico *et al.* 2001]. After internalization, the mesendodermal cell move away from the blastopore and the mesodermal and endodermal layers are formed eventually along the rostrocaudal embryonic axis [Warga *et al.* 1999] (Figure 1.4, [Montero *et al.* 2004]).

During vertebrate gastrulation movements, convergence and extension (CE) movements are most complicated and challenging because they represent a category of morphogenic process called “mass movement”. During CE, polarized cells elongate along the medio-lateral axis and undergo intercalation which leads to a medio-lateral narrowing (convergence) and an anterior-posterior lengthening (extension) of the forming embryonic axis [Keller *et al.* 1992]. In the recent time-lapse analyses of gastrulation in the fish *Fundulus heteroclitus* and zebrafish, it was reported that mediolateral cell intercalation in the axial gastrula appears to be the driving force for axial mesodermal CE movements [Trinkaus *et al.* 1992; Trinkaus 1998; Glickman *et al.* 2003]. In addition, the mediolateral distance towards the dorsal midline is another critical factor for the directional CE movements. For example, the mesodermal and ectodermal cells in the more distal domain are only slightly mediolaterally elongated and migrate slowly, individually and indirectly; while as the cells approach the midline, they are more elongated and directed with increased rates of movements towards the dorsal midline. As they reach the shield, the cells undergo a bipolar mediolaterally directed protrusion, leading to convergent extension of mesoendoderm along the anterior-posterior axis. Therefore, both mediolateral intercalation and directed migration contribute to the convergence and extension of tissues [Keller *et al.* 2000; Keller 2002] (Figure 1.4, [Montero *et al.* 2004]).

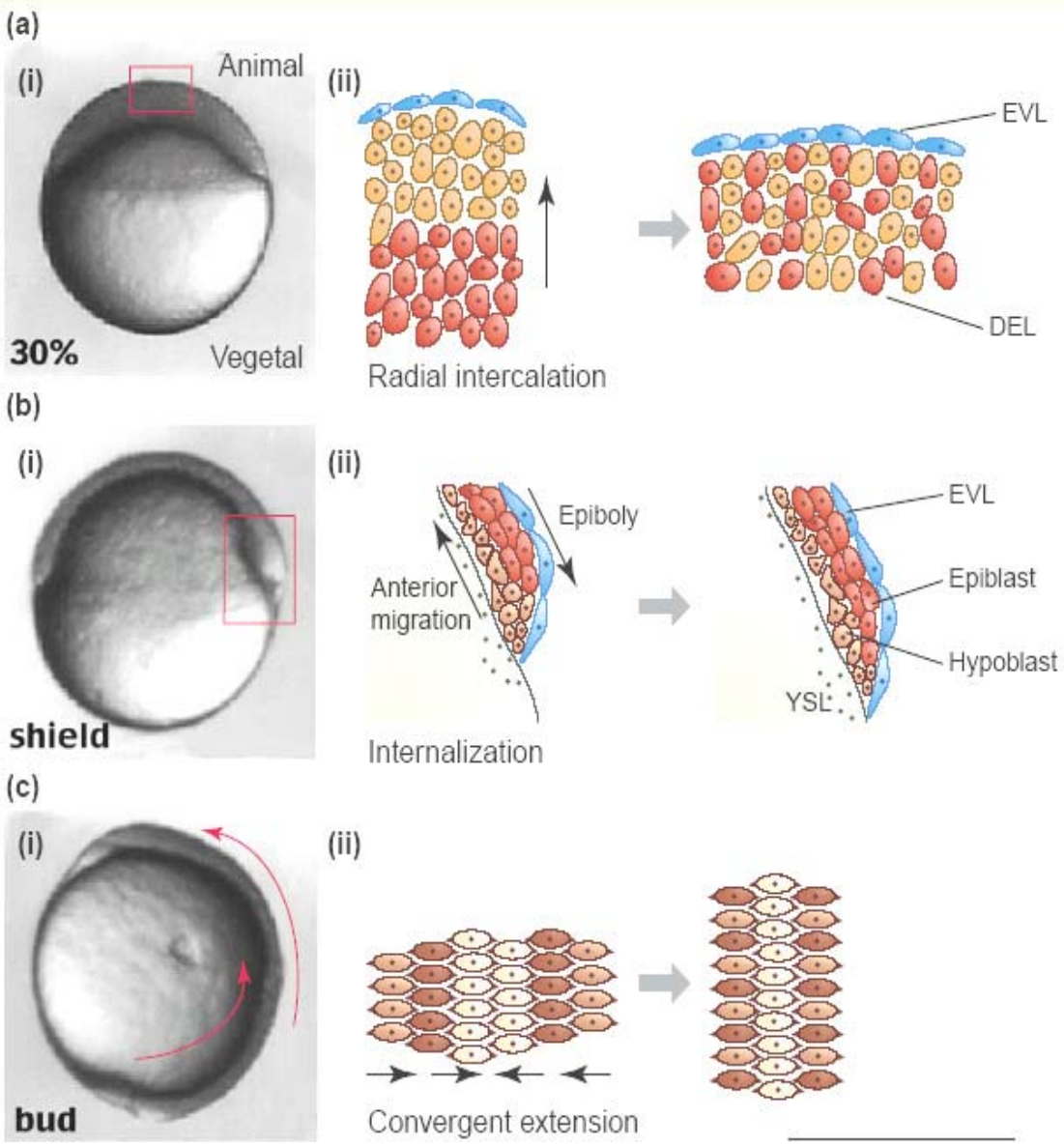

Figure 1.4 Gastrulation movements in zebrafish. (a) At the onset of gastrulation, radial cell intercalations of blastodermal cells drive epiboly movements. (b) At early stages of gastrulation, mesendodermal progenitor cells undergo internalization. (c) During gastrulation, mediolateral cell intercalations of mesendodermal cells drive axial mesodermal convergence and extension movements. Arrows indicate the directions of cell or tissue movements. (ii) is the schematical illustration of cell movements in the boxed area of (i). Adapted from [Montero *et al.* 2004]

1.2.2.2.2 Molecular mechanism underlying CE movements

Many molecules, especially those of the BMP, FGF and Wnt families, are known to play major roles in establishing cell fates and regulating cell movements during gastrulation. BMP family members are mostly responsible for cell fate determination, whereas FGF signaling and Wnt signaling have many functions both in cell fate determination and in morphogenesis [Myers *et al.* 2002; Leptin 2005]. Generally, the Wnt/*β*-catenin pathway, also referred as the canonical Wnt pathway, is mainly important for axis formation. However, it has also been reported to be essential for CE movements by activation of signal transducer and activator of transcription 3 (Stat3) which is required for the migration of mesodermal cells toward the dorsal midline during zebrafish gastrulation [Yamashita *et al.* 2002]. Besides, accumulating studies have demonstrated that non-canonical Wnt pathway, known as planar cell polarity (PCP) pathway in *Drosophila,* also plays pivotal role in the CE movements through the regulation of mediolateral cell polarization in vertebrates [Oates *et al.* 1999; Yeo *et al.* 2001; Kuhl 2002; Tada *et al.* 2002]. In *Xenopus*, deregulation of two noncanonical Wnt ligand genes, *wnt11* and *wnt5*, displayed CE movement defects without affecting cell fate. Consistently, the reduced CE movement phenotype was also observed in zebrafish *silberblick* (*slb*) /*wnt11* and *pipetail* (*ppt*)/*wnt5* mutants [Rauch *et al.* 1997; Heisenberg *et al.* 2000]. The Wnt11 (*slb)* mutant showed more anterior CE defects, whereas Wnt5a (*ppt)* functioned more in posterior regions [Rauch *et al.* 1997; Heisenberg *et al.* 2000]. However, they exhibited partially overlapping functions in regulating CE movements in lateral domains of the gastrula.

In *Drosophila*, Rho small GTPases including RhoA were reported to function downstream of Wnt11 in regulating planar cell polarization [Wolff *et al.* 1998]. Consistently, PCP pathway-mediated by RhoA has been reported to regulate convergence and extension movements in *Xenopus* gastrulation. For instance, RhoA can be activated by Wnt/Frizzled through Daam1, a novel Formin-homology protein, or through xNET1, a RhoA-specific guanine nucleotide exchange factor, during *Xenopus* gastrulation movements [Habas *et al.* 2001; Miyakoshi *et al.* 2004]. RhoA also mediates the non-canonical Wnt or *Xenopus* paraxial protocadherin (XPAPC) signals to control *Xenopus* CE movements by activation of ROKalpha, and/or JNK [Marlow *et al.* 2002; Unterseher *et al.* 2004; Kim *et al.* 2005]. In addition, the explanation of mesodermal cells in *Xenopus* reveals that both Rho and Rac modulate cell polarization, motility and protrusive activity in the trunk mesoderm, while Rho alone regulates the retraction of the lagging edge of the cell in the prospective head mesoderm [Ren *et al.* 2006] with distinct and overlapping roles during CE [Tahinci *et al.* 2003]. However, whether RhoA is involved in non-canonical signaling pathway to control convergence and extension movements and how the downstream effectors are involved in this process during zebrafish gastrulation has never been addressed.

1.2.2.3 Apoptosis in zebrafish

1.2.2.3.1 Apoptosis in normal development

In addition to the contribution of gastrulation movements to the embryonic morphogenesis through forming three germ layers and generating body axes, programmed cell death (apoptosis) is also critical for embryogenesis in the maintenance

of tissue homeostasis [Sanders *et al.* 1995; Jacobson *et al.* 1997; Vaux *et al.* 1999]. For better understanding of the significance of apoptosis in development, Ernst and Glucksmann classified and named the apoptotic pattern into three classes, morphogenetic apoptosis, histogenetic apoptosis and phylogenetic apoptosis [Glucksmann 1965]. Morphogenetic apoptosis occurs during the formation of cavity, in which the folding, bending, cavitation, fusion, or separation of tissues and cells are involved. In contrast, histogenetic apoptosis takes place after tissue is maturated or remodeled through the elimination of cells, such as the clearance of neurons that loses their function in connecting with their targets. Phylogenetic apoptosis happens to remove the unneeded structures or embryonic cells during evolution or maturation, such as the elimination of the mesonephros and pronephros or the tadpole's tail in adults in higher vertebrates. All three types of apoptotic patterns have been found in the normal development of vertebrates, including frog [Lamborghini 1987], chick [Garcia-Porrero *et al.* 1979; Ilschner *et al.* 1992], mouse [Young 1984], and rat [Kim *et al.* 1973; Pellier *et al.* 1994]. Consistently, in zebrafish, they also have been demonstrated to be important for embryonic development [Cole *et al.* 2001; Yamashita 2003].

1.2.2.3.2 Mechanism of apoptosis

The two best-studied mechanisms of apoptosis in vertebrates are the extrinsic (death receptor) and the intrinsic (mitochondrial) apoptotic pathways (Figure 1.5, [Pyati *et al.* 2007]). The extrinsic pathway is triggered upon the binding of extracellular ligands to their death receptors, such as the Fas ligand (FasL), tumor necrosis factor a (TNFa), or TNF-related apoptosis inducing ligand (TRAIL) binding to the Fas (CD95 or APO-1),

TNFa-, or TRAIL receptors, respectively. This activates the trimerization of the receptor, cytoplasmic adapter protein and Fas-associated death domain protein (FADD) to form a death-inducing signaling complex (DISC). Then, the DISC complex brings caspase-8 proenzymes into close proximity to cleave and activate each other, which subsequently leads to the proteolytic cleavage and activation of procaspase-3, and other important downstream procaspases such as procaspase-6, -8, and -10. Consequently, the amplified activation of caspases signaling inhibits or activates various downstream target proteins or ‘‘death substrates’’ including poly(ADP)-ribose polymerase (PARP), endonucleases, protein kinase Cd, and structural proteins such as the lamins, Gas2 and a-fodrin, and gelsolin. In contrast, the intrinsic pathway is activated by many biochemical factors including environmental insults, DNA damage, abnormal covalent binding of toxins to macromolecules, oxidative stress, and lipid peroxidation [Nicholls *et al.* 2000]. These stresses or developmental cues induce the activation of p53, which transcribes genes encoding BH3-only pro-apoptotic proteins. This increased expression of pro-apoptotic proteins overcomes the blockage from anti-apoptotic Bcl-2 family members and allows Bak and Bax to oligomerize, which leads to mitochondrial outer membrane permeabilization (MOMP) and subsequent releasing of Cytochrome C. The cytoplasmic Cytochrome C then induces a conformational change of the cytosolic adapter molecule Apaf-1, allowing Apaf-1 to recruit and activate multiple caspase-9 proenzymes, which in turn cleaves and activates caspase-3, leading to further downstream caspases activation. By a self-amplificatory way, the mitochondria seemingly play a critical role in the intrinsic apoptosis to amplify the apoptotic signals, which leads to more releasing of Cytochrome c, subsequently resulting in DNA fragmentation, chromatin condensation in

nucleus, and cell death eventually [Pyati *et al.* 2007]. The intrinsic and extrinsic apoptosis have been extensively studied in the *in vitro* cell culture system, their roles and the molecular mechanism in the *in vivo* system especially in zebrafish embryonic development need to be further elucidated.

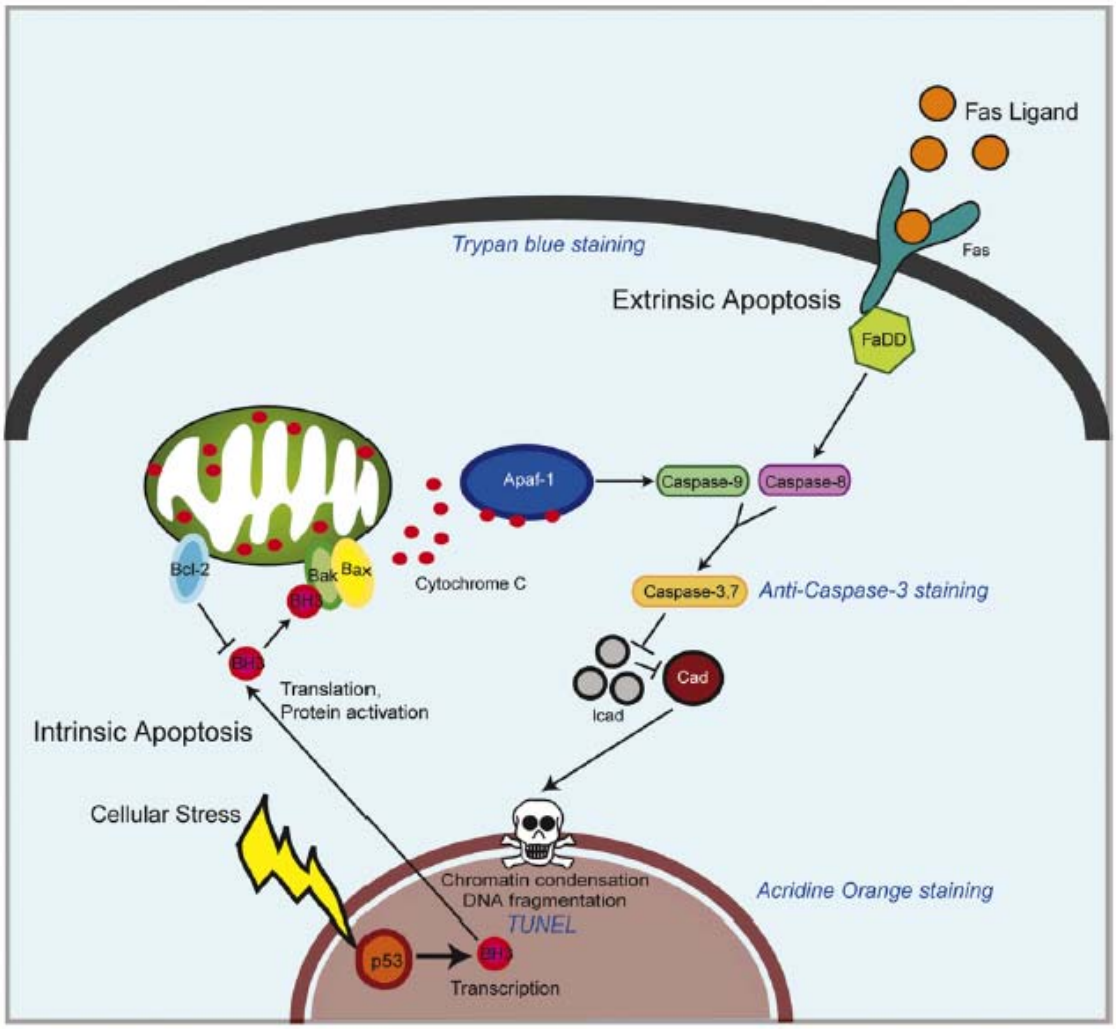

Figure 1.5 The extrinsic and intrinsic apoptotic pathways of in vertebrate. Adapted from [Pyati *et al.* 2007]. Please see detailed description in the section *1.2.2.3.2*.

1.2.2.3.3 Zebrafish as a powerful model for apoptosis study

Besides the advantages of zebrafish as a model system mentioned before (detail see 1.2.1), the evolutionarily conserved core components of apoptotic pathways in zebrafish further suggests that it could be a powerful model to study cell death *in vivo*. By bioinformatics analyses, the full-length, functional homologs of most mammalian Bcl-2 family members including anti-apoptotic *bcl-xl*, *bcl-2*, *mcl-1*, *nr13*, and *boo/diva*; proapoptotic *bax* and *bok*; and BH3-only genes *bid*, *bad*, *bmf*, *noxa*, *puma*, and *bik* have been found in zebrafish genome [Kratz *et al.* 2006]. Some of these homologs, such as *bcl-2* [Langenau *et al.* 2005], *mcl-1a* [Chen *et al.* 2000a], *bcl-xl* [Chen *et al.* 2001], *mcl-1* [Hong *et al.* 1999], *nrz* [Arnaud *et al.* 2006] have been cloned and characterized in zebrafish. Overexpression of fusion transgene of *bcl-2*–EGFP in T- and B-lymphoid cells have been shown to cause a 2.5-fold increase in thymocyte numbers and a 1.8-fold increase in GFP-labeled B cells in the kidney marrow by suppression of apoptosis [Langenau *et al.* 2005]. Consistently, the homologs of anti-apoptotic *mcl-1*, *zmcl-1a* and *zmcl-1b*, have been demonstrated to be important for maintaining the health of zebrafish embryos and preventing them from death ligand (DL)-induced apoptosis during early development, whereas the zebrafish pro-apoptosis protein, *bax1* and *puma*, play an essential role in irradiation-induced apoptosis [Kratz *et al.* 2006]. In addition to the *bcl-2* family members, other important components in the intrinsic apoptotic pathway, such as p53 and caspase 3, have also been characterized in zebrafish [Cheng *et al.* 1997; Thisse *et al.* 2000; Langheinrich *et al.* 2002]. Overexpression of caspase 3 in zebrafish embryos can induce extensive apoptosis and ceramide generation [Yabu *et al.* 2001a], while

antisense MO-mediated knockdown of p53 in flathead (fla) or Fancd2-deficient mutants leads to a striking rescue of developmental defects and restoration of normal cell survivals [Liu *et al.* 2003; Plaster *et al.* 2006]. Besides the above, more than 100 zebrafish mutants with altered apoptosis in diverse tissues, such as neural crest [Stewart *et al.* 2006], pituitary gland [Pogoda *et al.* 2006], notochord [Nowak *et al.* 2005], lateral line [Kozlowski *et al.* 2005], heart [Yuan *et al.* 2004; Aiyer *et al.* 2005], muscle [Bassett *et al.* 2003], blood [Paw *et al.* 2003; Ransom *et al.* 2004; Craven *et al.* 2005; Langenau *et al.* 2005], excretory system [Hammerschmidt *et al.* 1996; Pyati *et al.* 2006], germ cells [Koprunner *et al.* 2001; Weidinger *et al.* 2003; Ramasamy *et al.* 2006], and skin [Nowak *et al.* 2005], have been identified. Therefore, given the conserved function of core components in apoptotic pathways and the availability of various loss of function mutants, zebrafish is an excellent and powerful *in vivo* model to extend our understanding in the cellular and molecular basis underlying cell death during normal vertebrate development and a wide spectrum of human diseases including cancer and degenerative diseases.

1.3 Objectives

During early embryonic development, three germ layers, namely ectoderm, mesoderm and endoderm, are formed to shape the embryonic body through the process of gastrulation. In vertebrates, it involves four principal movements - epiboly, internalization of mesendoderm, convergence and extension (CE) [Keller *et al.* 2000]. Most of the pioneering studies for the vertebrate gastrulation movements have been performed in frog *Xenopus,* and shown to be largely conserved among vertebrates [Schoenwolf *et al.* 2000]. During CE, polarized cells elongate along the medial-lateral axis and undergo intercalation which leads to a medio-lateral narrowing (convergence)

and an anterior-posterior lengthening (extension) of the forming embryonic axis [Keller *et al.* 1992]. Such polarization and movements would require precise reorganization and regulation of the cytoskeleton network and cell adhesion.

In addition to the massive gastrulation movements in the shaping of embryonic body, programmed cell death is also critical for the morphogenesis in developing organism. In zebrafish and *Xenopus*, apoptotic program responses to the developmental cues or environmental insults from the onset of gastrulation. During zebrafish normal embryonic development, only few cell apoptosis are observed at the end of gastrulation, and increased apoptosis is detected in those tissues undergoing extensive morphological changes, such as CNS [Cole *et al.* 2001; Yamashita 2003]. However, the molecular mechanism underlying dynamic changes of apoptosis and CE movement in zebrafish are poorly understood.

RhoA plays pivotal roles in cytoskeletal rearrangement and gene transcription, which contributes to a wide variety of cellular events and developmental processes, including migration, adhesion, cytokinesis, proliferation, apoptosis, and morphogenesis [Van Aelst *et al.* 1997; Etienne-Manneville *et al.* 2002]. However, most of the studies on the role of RhoA in embryonic development have been done on *Drosophila* and *Xenopus*. Very little is known about the direct function of RhoA in zebrafish development in general or in the *in vivo* control of cell dynamics specifically. This is despite zebrafish being a well-established model for developmental studies, its increasing popularity as an alternative vertebrate model for human diseases, and its amenability to specific gene knockdown by MO and functional rescue, as well as treatment with pharmacological inhibitors [Kimmel *et al.* 1995; Amatruda *et al.* 2002; Neumann 2002; Beis *et al.* 2006].

Furthermore, many findings on the inhibition of RhoA functions *in vivo* are obtained primarily using inhibitors to block Rho-Rock signaling, or over-expression of dominant negative forms of Rho or ROCK, thus it could neglect the possibility of functional interference due to non-specific inhibition on other closely-related members [Coleman *et al.* 2004].

Therefore, the major objectives of this work were to: i) isolate the zebrafish *rhoA* gene and characterize its temporal and spatial expression profile in zebrafish embryogenesis and adult tissues; ii) explore the functional importance of *rhoA* during zebrafish embryonic development using its MOs-mediated specific functional knockdown; iii) investigate the signaling pathways involved in *rhoA*-dependent developmental processes *in vivo*.

In the next chapter, essential methods and materials for the study of functions of RhoA in zebrafish embryogenesis will be introduced.

Chapter 2 Materials and methods

2.1 Gene isolation and cloning

2.1.1 Polymerase chain reaction (PCR)

The *rhoA* specific upstream primer with start codon, named zebrafish *rhoA* F1: 5'GAATTCATGGCAGCAATTCGCAAGAA3', was designed based on the zebrafish EST sequence. Then, the zebrafish *rhoA* F1 and SK, the downstream primer of pBluscript SK+, were used to amplify the *rhoA* gene from an adult zebrafish cDNA library [Thisse *et al.* 1994]. Standard PCR was performed in a 50 µl reaction using the Perkin Elmer DNA thermal cycler Model 480 or 9600 (Perkin Elmer, USA). Each reaction included 5 µl of 10X PCR buffer (0.5 M KCl; 0.1 M Tris-HCl, pH 8.8; 15 mM MgCl2; 1% Triton X-100), 2.5 µl of 2 mM dNTP, 0.5 µl of 0.2 ug/µl sense primer, 0.5 µl of 0.2 ug/µl antisense primer, 0.2 µl of 5 U/µl Taq polymerase and 1 µl template DNA. A typical program used for amplifying 1 kb DNA product was as follows: denaturation at 94 °C for 5 minutes for 1 cycle, followed by 30 cycles of (denaturing at 94 °C for 30 seconds, annealing at 55 °C for 1 minute and extending at 72 °C for 1 minutes) and final extension at 72 °C for 10 minutes. The extension time was increased 1 min per 1 kb if the desired product was larger than 1 kb.

2.1.2 Rapid amplification of cDNA ends (RACE)

To amply full-length zebrafish *rhoA* gene including 5'UTR, coding region, and 3'UTR, 5' and 3' RACE was applied. The cDNA library was generated using SMART TM RACE cDNA Amplification kit (Clontech), which was used as template for both

5'RACEs and 3'RACEs. Combination of designed antisense gene-specific primers and commercial universal primers were applied for 5'RACEs, and combination of sense gene-specific primers and commercial universal primers for 3'RACEs. RACE PCR conditions were essentially the same as the standard one as described above, except that different annealing temperatures were used depending on the melting temperature (Tm) of the gene-specific primers.

2.1.3 Purification of PCR products

PCR products can be directly purified using QIAquick PCR purification kit, or recovered from gel after electrophoresis using QIAquick Gel Extraction Kit (Qiagen, USA). For QIAquick PCR purification kit, 5X volume of Buffer PB was added to 1X volume of PCR sample. Then mixture was transferred to the QIAquick spin column. After short spin for 1 minute, the flow-through was discarded. Then wash the spin column with 0.75 ml of Buffer PE and followed by centrifugation for 1 minute. Discarding the flow-through again, and purified PCR product was then eluted with 20-30 μl of sterile water or TE buffer by incubating for 1 minute and centrifuging for 1 minute. For QIAquick Gel Extraction Kit, the PCR band of interest was cut from the gel, melted at 50°C in Buffer QX1 for 10 minutes, and then loaded into a QIAquick spin column. The rest procedures were the same as purification using QIAquick PCR purification kit. The purified PCR product was used for cloning.

2.1.4 Cloning of PCR products

2.1.4.1 DNA ligation

The recovered PCR products were cloned into the pGEMT-vector system (Novagen, USA). DNA ligation reaction was carried out typically in a 20 µl volume, containing 2 µl of 10X ligation buffer (0.3 M Tris-HCl, pH 7.8; 0.1 M MgCl2; 0.1 M DTT and 5 mM ATP), insert DNA (PCR product), vector DNA (pGEMT-vector) and 1 unit T4 DNA ligase. The molar ratio of insert to vector DNA was usually 2:1 or 4:1. Ligation reaction was incubated at 4°C overnight. Subsequently, the ligation reaction was terminated by inactivating the ligase by heating at 80°C for 5 minutes and then transformation was carried out.

2.1.4.2 Preparation of competent cells

Successful cloning relies on high transformation efficiency. Normally $>10^7$ transformed colonies per µg of supercoiled plasmid is good for most cloning applications. For the preparation of competent bacteria cells, 2 ml of LB broth were incubated with a single fresh colony of *Escherichia coli* (*E.coli*) strain DH5α at 37°C with 250 rpm shaking overnight. On the following morning, 0.5 ml of the culture was re-inoculated into a 250 ml flask containing 50 ml of LB broth and shaken at 250 rpm at 37°C until OD600 reached around 0.5. The culture was chilled on ice for 15 minutes after being transferred into 50 ml Falcon tubes. Cells were pelleted by centrifugation at 1,000 g at 4°C for 15 minutes. The cell pellets were drained thoroughly and resuspended in RF1 (100 mM RbCl; 50 mM MnCl2; 30 mM Potassium acetate; 10 mM CaCl2 and 15% glycerol) with 1/3 rd volume of the original bacteria culture. After incubation on ice for 15 minutes, the cells were spun down and resuspended in 1/12.5 of the original volume of RF2 (10 mM MOPS; 10 mM RbCl; 75 mM CaCl2; 15% glycerol). After another 15 minute-incubation on ice, the competent cells were transferred into 1.5 ml microcentrifuge tubes in aliquot

and fast-frozen in liquid nitrogen. These aliquots can be stored at -80°C for several months.

2.1.4.3 Transformation

Normally 10 µl of ligation reaction was added into 100 µl of *E.coli* DH5α competent cells. This transformation mixture was then incubated on ice for 30 minutes. The mixture was heated at 42°C for 90 seconds and cooled immediately on ice for 2 minutes. 900 µl of LB medium was added to the mixture and incubated with shaking (200 rpm) at 37ºC for 1 hr. Alternatively, 800 µl of TSB and 20 µl of 1 M Glucose was added to the transformation mix and incubated with shaking at 37ºC for 1-1½ hrs. After incubation, 1/10 and 9/10 of the transformation reaction mixture was spread onto two separate LB plates supplemented with appropriate antibiotics in order to produce proper density of transformant colonies. The plates were incubated at 37°C overnight.

2.1.5 DNA sequencing

Automated sequencing reactions were carried out using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). The kit contains a sequencing enzyme AmpliTaq® DNA Polymerase called FS and a set of dye labeled terminators for fluorescent cycle sequencing larger fragments with more accuracy. Each sequencing reaction (20 µl) contains 8 µl of Terminator Ready Reaction Mix, 200-500 ng of double strand DNA, and 1 µl of primer (0.2 µg/µl). PCR was performed on the GeneAmp PCR System 9600 (Perkin Elmer) or Peltier Thermal cycler PTC200 (MJ Research, USA) with 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes, and finally hold at 4°C. Ethanol precipitation was carried out to purify the

extension products. 2 µl of 3 M NaOAc (pH 4.6) and 50 µl of 95% ethanol was mixed with the 20µl of reaction mix, and incubated at room temperature for 15 minutes. The tube was spun at 4°C for 20 minutes at 14,000 rpm. The pellet was rinsed with 250 µl of 70% ethanol and air-dried. The DNA pellet was dissolved in 6 µl of loading dye [50 ml contains EDTA (25 mM, pH8.0) 1 ml; 10 ml deionised formamide; 50 mg Dextran blue and 39 ml H_2O] and heated at 92°C for 3 minutes. Samples were then chilled on ice for 2 minutes before being loaded into the sequencing gel (18g urea; 5 ml 10X TBE; 5 ml long range gel solution and 26 ml H_2O; 250 µl 10%APS and 35 µl TEMED). The electrophoresis was carried out at 1,690 volts for 5-9 hours. The sequencing ladders were analyzed automatically by an ABI377 sequencer system and software.

2.2 Gene expression analysis

2.2.1 RNA expression

2.2.1.1 Isolation of total RNA from tissue or embryos

Total RNAs from different stages of zebrafish embryos and different tissues were extracted using TRIzol reagent (Gibco BRL). Briefly, about 50-200 embryos or 100 mg of tissues were quickly frozen in liquid nitrogen and homogenized in 1 ml of TRIzol reagent. The homogenate was incubated at room temperature for 5 minutes to allow nucleoproteins to dissociate before 200 µl of chloroform was added in. The mixture was shaken by hand vigorously for 15 seconds and incubated at room temperature for another 3 minutes, then centrifuged at 14,000 rpm for 15 minutes at 4°C to separate aqueous and organic phase. 500 µl of aqueous phase was then transferred to a new tube and an equal

volume of isopropanol was added. RNA was pelleted by centrifugation at 14,000 rpm for 10 minutes at 4°C and washed with 1 ml of 70% ethanol. The RNA pellet was then dissolved in 20 µl of DEPC (Diethyl pyrocarbonate) water and stored at -80°C.

2.2.1.2 Measurement of RNA concentration

RNA was quantified by optical density reading at 260 nm and 280 nm using UV-1601 spectrophotometer (Shimadzu, Japan). One unit of OD 260 is equivalent to 40 µg/ml of RNA. The ratio of OD 260: OD 280 at 1.8-2.0 indicates good quality of RNA products.

2.2.1.3 RNA gel electrophoresis

10 µg of total RNA was fractionated on 1.2% denaturing agarose gel (1.2% agarose, 1X MOPS, 6% formaldehyde). Each RNA sample contained 50% formamide, 1X MOPS, 7% formaldehyde and 0.1 mg/ml ethidium bromide and was heated at 65°C for 10 minutes before loading with loading buffer (1X 0.4% bromophenol blue, 6% sucrose in water). The gel was run at 80 volts in running buffer containing 1X MOPS and 3% formaldehyde until the dye runs out into the buffer. The gel was then rinsed in distilled water for photo-taking or Northern analysis.

2.2.1.4 Northern blot

After the agarose gel electrophoresis, RNA samples were transferred to Hybond™-N nylon membrane (Amersham, UK) overnight in 20X SSC. The membrane was then air-dried and cross-linked by UV irradiation on a 312 mm UV box for 3 minutes.

2.2.1.4.1 Prehybridization

Prehybridization was conducted to prevent non-specific hybridization of the probe to the membranes. The denatured salmon sperm DNA acted as a blocking reagent and helped to reduce the background signal. The membranes were placed in a hybridization-rolling bottle (HB-OV-BS, Hybrid) with the RNA side facing inwards. The bottle contains 5 ml of hybridization buffer (50% formamide; 5X Denhardt's solution; 4X SET; 0.2% NaPPi; 25 mM phosphate buffer; 0.5% SDS, 100 μg/ml denatured salmon sperm DNA and 10% w/v dextran sulfate). Salmon sperm DNA stock (10 mg/ml) was denatured in boiling water for 5 minutes and then kept on ice for 5 minutes. The bottle was then transferred to a hybridization incubator (Mini Oven MKII, Hybrid). Prehybridization was carried out at 42 °C for more than 2 hours with a spinning speed of 7 rpm.

2.2.1.4.2 Hybridization

Dig-labeled probe was denatured at 92 °C for 5 minutes in a heat-block (type 17600, Thermolyne, USA) and then immediately chilled on ice for 5 minutes. The Hybridization bottle was added with the chilled probe to the final concentration of 0.5 - 1X 10^6 cpm/ml buffer and swirled to mix the probe thoroughly. The bottle was re-capped and incubated at 42 °C for 16 hours.

2.2.1.4.3 Post hybridization wash

After hybridization, the buffer was discarded and 20 ml of washing solution (2X SET; 0.2% NaPPi and 0.5% SDS) was added. The hybridization bottle was agitated by gentle shaking at room temperature for 15 minutes. This washing step was repeated once. Afterwards, the following two washing steps were performed at 65 °C in the

hybridization incubator for 20 minutes each. Pre-warmed wash solution was used and the incubator was preset at 65 °C. A final stringent wash was carried out using a final wash solution (0.2X SET; 0.5% SDS) at 65 °C for 20 minutes. This was conducted only if the radioactivity count was still too high.

2.2.1.4.4 Autoradiography

The membrane was wrapped with a Saran polyethylene to keep the membrane moist. Once the membrane dries, it cannot be stripped and re-probed since the probe will be retained permanently on the blot. A Xomat (Kodak, USA) film was placed against its RNA side and then was put in an X-ray cassette with an intensifying screen at -80 °C for 4 h - overnight. All film handling was performed in the dark room. The autoradiogram was developed using the M35 Xomat developer (Kodak, USA).

2.2.1.5 Reverse-transcriptase PCR (RT-PCR)

RT-PCR was performed in either two-step reaction or one-step reaction. In two-step reaction, first step involved synthesis of first strand cDNA and the second step involved amplification of zebrafish *rhoA* and actin from single strand cDNA as template with gene specific primers for either zebrafish *rhoA* (forward (5'GAATTCATGGCAGCAATTCGCAAGAA3' and reverse (5'TCACAGCAGACAGCATTTGTT3') or *β-actin* (forward 5'-CCGTGACATCAAGGAGAAGCT-3', and reverse 5'-TCGTGGATACCGCAAGATTCC-3'). The first strand cDNA was synthesized in 30 µl of reaction buffer containing 3 µl of 10X first-strand buffer (50 mM Tris-HCl, pH8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT), 3 µl of 10 mM dNTP, 1 µl of RNAse inhibitor

(40U/µl), 3 µl of oligo dT primer (1µg/µl), 5 µg of total RNA, and 1 µl of MMLV reverse transcriptase (50U/µl). After incubating at 37°C for 1.5 hours, the reaction can be stored at -80°C or used as template for PCR immediately. PCR reaction was carried out using the standard condition described above. The one-step reaction was done using a Qiagen one-step RT-PCR kit (Qiagen, USA). The reaction mix contains all the components including a pair of gene specific primers as recommended by the kit specifications.

2.2.1.6 In situ hybridization

2.2.1.6.1 Synthesis of labeled RNA probe

1 µg of linearized DNA was used as template to synthesize the DIG labeled probe. The reaction was performed at 37°C for 2 hours in a total volume of 20 µl containing 4 µl of 5X transcription buffer (Stratagene, USA), 2 µl of DIG-NTP mix [10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP and 3.5 mM DIG -UTP (Boehringer Mannheim, Germany)], 1 µl of RNase inhibitor (40U/µl) (Promega, USA) and 1µl of T7 RNA polymerase (50 U/µl) (Promega, USA). Following the reaction, 2 µl of RNase-free DNase I was used to digest the DNA template at 37°C for 15 minutes. 1 µl of 0.5M EDTA (pH 8.0) was used to stop the digestion. Subsequently, 2.5 µl of 4 M LiCl and 75 µl of cold pure ethanol were added to precipitate the RNA. After washing with 75% ethanol, the RNA probe was resuspended in 60 µl of DEPC treated water and cleaned using a Chroma Spin-100 DEPC H_2O Column (Clontech, USA) by centrifuging at 700 g for 5 minutes to remove the impurity and small RNA fragments. Following probes were used: zebrafish *rhoA* 3'UTR, *gsc* [Stachel *et al.* 1993], *ntl* [Schulte-Merker *et al.* 1994], *hgg1* [Akimenko *et al.* 1994], *dlx3* [Akimenko *et al.*

1994], *papc* [Yamamoto *et al.* 1998], *eve1* [Joly *et al.* 1993], *chordin* [Sasai et al. 1994], *bmp4* [Fainsod *et al.* 1994], *shh* [Krauss *et al.* 1993], *pax2*.1 [Krauss *et al.* 1991] and *myoD* [Weinberg *et al.* 1996].

2.2.1.6.2 Preparation of zebrafish embryos

Different stages zebrafish embryos were fixed in 4% paraformaldehyde (PFA)/PBS (0.8% NaCl, 0.02% KCl, 0.0144% Na_2HPO4. 0.024% KH_2PO4, pH 7.4) for 12 to 24 hours at room temperature or 4°C. After fixation, the chroins were remonved from embryo. Then the embryos were washed in PBST (0.1% Tween 20 in PBS) twice for 1 minute each, followed by four times for 20 minutes each on a nutator (ClAY ADAMS® Brand, Becton Dockinson, USA) at room temperature. After changing PBST to methanol, the embryos were kept at -20°C for several months. Before they were used for in situ hybridization, the embryos were rehydrated in PBS in two or three times by changing half volume of solution each time. For embryos older than 14 somites (>16 hpf), proteinase K (10 μg/ml) treatment is necessary. The time of exposure depended upon embryos age and the specific activity of proteinase K, which varied from batch to batch. For most cases, the conditions used are as given. 16-24 hpf 3-4 minutes; 24-32 hpf 5-6 minutes; 32-50 hpf 10-20 minutes. To stop the reaction, the proteinase K solution was removed completely, and the embryos were fixed again in 4% PFA/PBS for 20 minutes at room temperature. Embryos were first washed in PBST twice for 1 minute and then 4-5 times for 15-20 minutes each.

2.2.1.6.3 Prehybridization

Prehybridization was performed by changing half volume of washing solution with hybridization buffer [50% formamide, 5X SSC, 50 µg/ml Heparin, 500 µg/ml tRNA, 0.1% Tween 20, pH 6.0 (adjusted with bycitric acid)] and incubated at room temperature for 1 hour. This solution was removed and replaced with hybridization buffer; embryos were incubated at 68°C for 5-10 hours.

2.2.1.6.4 Hybridization

1-2 µl of DIG-labeled probe was diluted in 200 µl of hybridization buffer. The probe was denatured by heating at 80°C for 5 minutes followed by 2 minutes of ice bath. Embryos of different stages were selected and placed in one tube or separate tubes depending on the experimental conditions. The original buffer was replaced with the denatured probe dissolved in hybridization buffer. Hybridization was performed at 68°C in a circulating water bath overnight with shaking.

2.2.1.6.5 Post-Hybridization washes

The next day, the probe was removed and replaced with pre-warmed 100% hybridization wash solution (hybridization buffer without tRNA and heparin) for 15 minutes. The embryos were then washed in the following order of wash solutions, 75% hybridization wash solution (25% 2X SSCT (SSC with 0.1% Tween 20)), 50% hybridization wash solution (50% 2X SSCT), 25% hybridization wash solution (75% 2X SSCT) for 15-20 minutes each. This was followed by washing with 2X SSCT twice for 30-45 minutes each and 0.2X SSCT twice for 30-45 minutes each. Subsequently, the embryos were washed twice with PBST (PBS with 0.1% Tween 20) at room temperature for 5 minutes each.

2.2.1.6.6 Antibody incubation

2.2.1.6.6.1 Preparation of pre-absorbed DIG antibody

Commercial DIG-AP antibodies (Boehringer) should be pre-incubated with biological tissues or embryos, preferably of the same origin as the sample used for hybridization, in order to decrease the staining background and increase signal-to-noise ratio. Anti-DIG-AP was diluted to 1:500 and 1:50 in Maleic Acid buffer (0.15 M Maleic acid, 0.1 M NaCl; pH 7.5)/10% FCS (Fetal calf serum, Gibco BRL) respectively and incubated with 50 zebrafish embryos of any stages on a nutator at 4°C overnight. After that, the antibodies solution was transferred to a new tube and further diluted 10X with Maleic Acid buffer/10% FCS. 10 µl of 0.5 M EDTA (pH 8.0) and 5 µl of 10% sodium azide were added to prevent bacterial growth. The pre-absorbed antibody was stored at 4°C and can be used for many times.

2.2.1.6.6.2 Incubation with pre-absorbed antibodies

The embryos after hybridization and post hybridization washes were incubated in Maleic Acid buffer/10% FCS for 2 hours at room temperature to block non-specific binding sites for antibody. After removing the blocking solution, the embryos were incubated with pre-absorbed anti-DIG-AP antibody at 4°C overnight.

2.2.1.6.7 Color development

Embryos were washed in PBST twice for 1 minute each, and 4 times for 15-20 minutes each on a nutator at room temperature followed by washing in buffer 9.5 (0.1 M Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 10 mM NaCl and 0.1% Tween 20) once for 30 seconds and twice for 10 minutes each. 4.5 µl of NBT (Nitroblue tetrazolium, Boehringer

Mannheim, 50 mg/ml in 70% dimethyl formamide) and 3.5 µl of BCIP (5-bromo, 4-chloro, 3-indodyl phosphate salt, Boehringer Mannheim, Germany; 50 mg/ml in H_2O) was added into 1 ml of buffer 9.5 with embryos and mixed thoroughly. Embryos were kept in dark at room temperature for few minutes to several hours, and the progress of staining was monitored from time to time under a Leica MZ12 microscope (Leica, Germany). To stop the reaction, staining solution was removed and the embryos were washed in 1X PBST twice for 10 minutes each. Embryos can be preserved in 4% PFA/PBS at 4°C.

2.2.1.6.8 Mounting and photography

Selected embryos were washed with PBST twice for 10 minutes each and transferred to 50% glycerol/PBS, equilibrated at room temperature for several hours. For whole mounts, a single chamber was made by placing stacks of 3-5 small cover glasses on both side of a 25.4X76.2 mm microscope slide. Small cover glasses in the stacks will be perfectly solid 1 hour after placing a drop of Permount between them. Selected embryo was transferred to the chamber in a small drop of 50% glycerol/PBS and oriented by a needle. A 22X44 mm cover glass with a small drop of the same buffer was superimposed onto the embryo. The orientation of the embryo can be adjusted by gently moving the cover glass. For flat specimen, the yolk of selected embryo was removed completely by needles. The embryo without yolk was then placed onto a slide with a small drop of 50% glycerol/PBS and adjusted to a proper orientation by removing excess of liquid and with the help of needles. A small fragment of cover glass (a bit larger than the specimen) was covered onto the embryo. Care was taken to avoid bubbles and a drop of 50% glycerol/PBS was added to fill the space under the cover glass. This specimen

was sealed with nail polish along the edge of the cover glass to prevent it from drying. Photographs were taken using a camera mounted to an Olympus AX-70 microscope (Olympus, Japan). The films used were Kodak Gold 200 and 400 ASA.

2.2.1.7 Cryosection of embryos

2.2.1.7.1 Preparation of slides and blocks

The fixed and stained embryos were first transferred into molten 1.5% agar, equilibrated with 30% sucrose (at 48ºC) in a detached cap of eppendorf tube. The samples were adjusted to the required orientation with needles before the agar solidified. After the agar block solidified, a small block was cut with razor or blade in such a way that a flat base and a slanting top edge was created for proper positioning and sectioning of the sample. The block was then transferred to 30% sucrose solution and incubated at 4ºC overnight for equilibration.

2.2.1.7.2 Sectioning, mounting and photographing

Subsequently, the block was placed on the frozen surface of a layer of tissue freezing medium cryostat (Reichert-Jung, Germany) on the pre-chilled tissue holder. The block was then coated with a drop of cryostat freezing medium and frozen in liquid nitrogen until the block had solidified completely. The frozen block was placed in the cryostat chamber (Reichert-Jung, Germany) for 30 minutes to 1 hour to equilibrate with chamber temperature of –25ºC. Normally, 10 μm thick sections were cut and placed on superfrost plus slides (Fisher, USA). The slides were dried on a 42ºC hot plate for about 30 minutes to 1 hour. The sections were then fixed briefly with 4% PFA-PBS for 10 minutes and washed gently with PBS for 3 times, 10 minutes each. These sections was

either embedded in several drops of glycerol and covered with glass cover-slip for photography or used for section *in situ* hybridization.

2.2.2 Protein analysis

2.2.2.1 Extraction of protein

The pooled embryos injected with *rhoA* MO or control MO were dechorioned and deyolked manually at 18-somite stage, and lysed in T-PER reagent (BioRad, USA). 100 µl of T-PER and 1 µl of proteinase inhibitor (100 X stock) was added to every 200 embryos and homogenized well with a hand-held homogenizer (Sigma, USA). The homogenized sample was then centrifuged at 10,000 rpm for 5 minutes. The supernatant containing the total protein was collected and stored at –80ºC until further use.

2.2.2.2 Estimation of protein concentration

Concentration of the protein extracted from samples was estimated using Protein Assay Reagents (BioRad, USA). Initially the standard curve was plotted using different concentration of protein standards (0.1 µg/µl – 1.0 µg/ml). 1 µl of a particular concentration standard was mixed well with 800 µl of sterile water and 200 µl of Protein Assay Dye Reagent concentrate (BioRad, USA) and optical density read in a spectrophotometer at 595 nm. Graph was plotted with concentration of standard on X-axis and OD 595 readings on the Y-axis. The samples were similarly mixed with 800 µl of sterile water and 200 µl of Protein Assay Dye Reagent concentrate and then measured at 595 nm. The corresponding protein concentration of the samples was estimated by extrapolating against the standard graph. In some cases, the protein samples were

required to be concentrated because of low initial concentration. The samples can then be stored at −80ºC until further use.

2.2.2.3 SDS-PAGE gel electrophoresis

The proteins were analyzed on 12.5 % or 10 % SDS polyacrylamide gels with a Mini Protean II electrophoresis apparatus (Bio-Rad Laboratories, California, USA). The gels were cast with 1.5 mm spacers and ten-well combs (Bio-Rad Laboratories, California, USA). The resolving gel contained 12.5% or 10 % (w/v) acrylamide, 0.48 % (w/v) N-N'-methylbisacrylamide, 0.375 mM Tris-HCl pH 8.8, 0.1 % 40 (w/v) SDS, 0.0075 % (w/v) AMPS and 0.05 % (v/v) TEMED. After the resolving gel was poured into the gel cast, it was leveled with Milli-Q water. When the resolving gel had polymerized, the top edge of the gel was rinsed three times with Milli-Q water. The stacking gel which contained 4 % (w/v) acrylamide, 0.133 % (w/v) N-N'-methylbisacrylamide, 0.125 mM Tris-HCl pH 6.8, 0.1 % (w/v) SDS, 0.0075 %(w/v) AMPS and 0.08 % (v/v) TEMED was pipetted onto the top of the resolving gel. 100 µg of protein extract was mixed with 1/5 volume of 6 × loading buffer [0.2 M Tris-HCl pH 6.8, 25 % (v/v) glycerol, 25 % (v/v) SDS, 12.5 % (v/v) 2-mercaptoethanol, 0.005 % (w/v) bromophenol blue] and boiled for 4 min at 85 °C. Electrophoresis was performed at 50 mA/gel for 1 hr at room temperature in SDS-running buffer [25 mM Tris, 192 mM glycine and 0.1 % (w/v) SDS].

2.2.2.4 Western blotting

After transferring, blots were incubated with blocking buffer (3% BSA in TBST) for 3h at room temperature or 4 °C overnight. Blots were then probed with primary

antibody diluted in Blocking buffer for 60 min at room temperature followed by 10 min washing with 0.1 % Tween 20 in PBS for three times. After that, blots were incubated with secondary antibody diluted in PBS with 0.1% Tween 20 for 60min at room temperature and followed by three times washing in PBS with 0.1% Tween 20. Blots were then subjected for detection with ECL kit.

2.3 Functional study

2.3.1 Maintenance and breeding of zebrafish

Wild-type Zebrafish (*Danio rerio*) were purchased from a local supplier and raised under standard laboratory conditions [Salas-Vidal *et al.* 2005]. The embryos were collected by two techniques. On the day before embryo collection, a clean tray layered with clean marbles was placed on the bottom of the tank. Sometimes multiple trays were used depending on size of tank and number of fishes. The following morning embryos were collected by siphoning with a plastic pipe. Alternative method of breeding and spawning of zebrafish used 2-tank system (inner breeding tank and outer embryo collection tank), one tank placed inside another with the inner tank having a mesh bottom and sufficient distance between the bottom of inner and outer tank. Adult male and female zebrafish were segregated by a plastic divider and placed in the inner tank the day before spawning. Some plastic green plants available from the local aquarists were introduced into the breeding tank to simulate natural environment. The next morning the divider was removed and the fishes were allowed to breed and spawn. The embryos were collected from the bottom of outer tank by using a mesh sieve and grown at 28°C. The required developmental stages were presented as hours post-fertilization (hpf).

2.3.2 Synthesis of 5' capped mRNA

5' capped mRNA was synthesized by mMessage Machine™ Sp6 or T7 kit (Ambion, USA). The typical reaction volume is 20 µl, containing 1 µg DNA linearized at 3' end of the clone for sense RNA, 2 µl 10X Reaction Buffer, 10 µl 2X NTP/Cap, 2 µl Enzyme Mix and nuclease-free water. The reaction was incubated at 37°C for 2 hours. After that, 1 µl RNase-free DNase I was added and mixed well. The tube was incubated for 15 minutes at 37°C. The recovery of RNA was performed by LiCl precipitation. First, 30 µl Nucleasefree water and 25 µl Lithium Chloride Precipitation Solution were added into the reaction mix. Then the reaction was chilled at -20°C for 30 minutes and centrifuged at 14,000 rpm, 4°C for 15 minutes. The pellet was washed by 250 µl 70% ethanol and re-centrifuged at 14,000 rpm for another 5 minutes. Finally, RNA was resuspended with DEPC treated water and 1 µl of RNasin® (Promega, USA) was added to prevent degradation. The RNA sample can be stored at -80°C for 1 year.

2.3.3 Morpholinos preparation

Two non-overlapping anti-sense MOs against 5'UTR (*rhoA* MO1: 5'-TCCGTCGCCTCTCTTATGTCCGATA-3') or translation start-site of zebrafish *rhoA* gene (*rhoA* MO2: 5'-CTTCTTGCGAATTGCTGCCATTTTG-3'), one splicing MO targeting the *rhoA* splice donor site of exon 3 (E3I3 MO: 5'-ACACCAAAGAGCATTCTTACTAAAC-3'), one 5-bp mismatch MO of *rhoA* MO1 (5'-TCgGTCcCCaCTCTaATGTCgGATA-3'; mismatches in lower case), one anti-sense MO against zebrafish *wnt5* gene (*wnt5* MO: 5'-GTCCTTGGTTCATTCTCACATCCAT-3'), and another one against zebrafish *wnt11* gene (*wnt11* MO: 5'-GAAAGTTCCTGTATTCTGTCATGTC-3'), as well as one standard control MO (5'-

CCTCTTACCTCAGTTACAATTTATA-3') were synthesized by Gene Tools (Gene Tools, Inc.). All the MOs were resuspended in 1x Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM $Ca(NO3)_2$, and 5.0 mM HEPES pH 7.6), and injected into embryos at one-cell stage. For effective functional knockdown, the dose of MOs injected per embryo are as follows, *rhoA* MO1 (2.4 ng), *rhoA* MO2 (5.7 ng), E3I3 MO (5.7 ng), 5-bp mismatch MO (5.7 ng), and standard control MO (5.7 ng). In the combination of gene suppression with inhibitors treatment, 1.15 ng of *rhoA* MO1 or control MO was applied per embryo. For the rescue experiments, capped full length mRNA encoding zebrafish *rhoA* (PCS2+-*rhoA*), mouse prototype *Mek1* (pXJ40-*mMek1*) and human *BCL-2* (pRCCMV-hBCL-2) were synthesized *in vitro* using the mMessage mMachine Kit (Ambion, USA). Each of these mRNAs was titrated and co-injected with *rhoA* MO1 (2.4 ng) at one-cell stage. The sub-optimal dose for *rhoA, Mek1* or *BCL-2* mRNA that could effectively prevent RhoA knockdown defects was 11.25 pg, 60 pg and 5 pg, respectively.

2.3.4 Microinjection into embryos

The needles used for the microinjection were prepared using optimized conditions of heat and pull time for different purposes using the Sutter Micropipette puller P-97 (Sutter Instruments Co, USA). RNAs, plasmid DNA and antisense oligos were injected into the cytoplasm of 1-cell stage zebrafish embryos using WPI's microprocessor-controlled Nanoliter 2000 (World Precision Instruments, Inc., USA) by placing the embryos under a dissection microscope (Olympus SZX12). Each embryo received 2.3 nl of the samples for one injection. The injected embryos were reared in egg water (1 ml of egg water contains 10% NaCl, 0.3% KCl, 0.4% $CaCl_2$, 1.63% $MgSO_4.7H_2O$, 0.01% methylene blue, and 95 ml ddH_2O).

2.3.5 Treatment with pharmacological inhibitors

Mek inhibitor (U0126, Promega), Jnk inhibitor (SP600125, Calbiochem), and Bcl-2 inhibibtor (HA 14-1, Calbiochem) were dissolved in dimethyl sulfoxide as 10 mM stock solution. They were titrated on wild type embryos before applied on morphants, e.g. U0126 (5-40 μM), SP600125 (0.3-1.2 μM), and HA 14-1 (0.5-2.5 μM). The optimal concentrations were chosen whereby the corresponding pathways could be effectively inhibited without generating excessive and non-specific global defects (suggesting no toxicity). Embryos at 2.5 hpf were emerged into the egg water with inhibitor and continuously incubated till 15-18 somites stage, then they were either collected for Western analysis and microscopy or fixed for apoptosis assay.

2.3.6 TUNEL assay

TUNEL assay (terminal deoxynucleotidyl transferase-mediated deoxyuridin triphosphate nick-end labeling) was performed using the DNA Fragmentation Assay Kit (Clontech, USA) to detect apoptotic cells. The preparation of embryos were followed the same protocol as that for *in situ* hybridization (details see section 2.2.1.6.2.). For better permeablization, embryos were immersed in aceton for 5 minutes at -20°C, followed by 3 times' washing with PBST for 5 minutes each at room temperature. Embryos were then equilibrated in 100 μl of Equilibration Buffer for 10 minutes at room temperature. After removing all the solution, embryos were incubated with enzyme mix (45 μl of Equilibration Buffer, 5 μl of Nucleotide Mix, 1 μl of TdT Enzyme) in dark for 2 hours at 37°C degree. To terminate the tailing reaction, embryos were incubated in 2X SSC at room temperature for 15 min and followed by 3 times washing with PBST for 5 minutes

each. Because the dUTP has already been label with fluorescein at the free 3'-hydroxyl ends, embryos were ready for photography.

2.3.7 Statistical analysis

To check for the effectiveness of knockdown by MOs, rescue by mRNAs, or inhibition by pharmacological inhibitors, the percentage of normal or defected embryos in respective treatments were compared. The data are presented as means ± s.d.. Each experiment was repeated at least three times. The statistically significant differences in mean values were assessed with the two populations (paired) *t*-test (Origin Pro 6.1).

Chapter 3 The role of RhoA in convergence and extension movements during zebrafish gastrulation and tail formation

Gastrulation shapes the early embryos by forming three germ layers, ectoderm, mesoderm and endoderm. In vertebrates, this process requires massive cell rearrangement including convergence and extension (CE) movements that involve narrowing and lengthening of embryonic tissues as well as cell elongation. Such polarization and movements require precise reorganization and regulation of the cytoskeleton network and cell adhesion. Rho small GTPases are key regulators for actin cytoskeleton dynamics. However, the signaling mechanisms underlying their functions in CE remain to be further elucidated. Here, we report the isolation and analysis of the expression of rhoA gene and functional role of rhoA during early embryonic development in zebrafish, and confirm by morpholino-based specific functional knockdown of RhoA protein and mapping of specific gene markers that rhoA positively regulates CE movement without affecting cell fates determination during zebrafish gastrulation as well as the tail development. By functional rescues, we extend our findings that RhoA also acts potently downstream of Wnt5 and Wnt11 and it involves at least the mDia-equivalent as well as the Rho kinase as the downstream effectors during the CE movements.

3.1 Results

3.1.1 Isolation of full length sequence of *rhoA* cDNA

To examine functions of *rhoA* during vertebrate development, we first cloned the *rhoA* gene from zebrafish. Zebrafish *rhoA* gene contains a 582-bp open reading frame, a 132-bp 5′UTR and a 650-bp 3′UTR (AY224600), and it is predicted to encode a 193-amino acid protein that is highly conserved among human, mouse, *Xenopus*, and Fugu (Figure 3.1a). The divergence at C-terminus provides evidence for the phylogenetic relationship among different species for this protein family. As shown in Figure 3.1a and b, the gene we isolated shares the highest identity (99%) with the zebrafish Rhoab identified through genomic annotation [Salas-Vidal *et al.* 2005], while Fugu, Human and Mouse RhoA have almost the same sequence at their C-terminus. In addition, divergence at the C-terminus among different members specifies unique homologs within the Rho subfamily. Based on this, we believe that the gene isolated here represents zebrafish *rhoA* not *rhoB* or *rhoC*.

a

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Zebrafish_RhoA   MAAIRKKLVIVGDGACGKTCLLIVFSKDQFPEVYVPTVFENYVADIEVDSKQVELALWDT 60
Zebrafish_Rhoab  MAAIRKKLVIVGDGACGKTCLLIVFSKDQFPEVYVPTVFENYVADIEVDSKQVELALWDT 60
Fugu_RhoA        MAAIRKKLVIVGDGACGKTCLLIVFSKDQFPEVYVPTVFENYVADIEVDSKQVELALWDT 60
Human_RHOA       MAAIRKKLVIVGDGACGKTCLLIVFSKDQFPEVYVPTVFENYVADIEVDGKQVELALWDT 60
Human_RHOB       MAAIRKKLVVVGDGACGKTCLLIVFSKDEFPEVYVPTVFENYVADIEVDGKQVELALWDT 60
Human_RHOC       MAAIRKKLVIVGDGACGKTCLLIVFSKDQFPEVYVPTVFENYIADIEVDGKQVELALWDT 60
Mouse_RHOA       MAAIRKKLVIVGDGACGKTCLLIVFSKDQFPEVYVPTVFENYVADIEVDGKQVELALWDT 60
Xenopus_RhoA     MAAIRKKLVIVGDGACGKTCLLIVFSKDQFPEVYVPTVFENYVADIEVDGKQVELALWDT 60
                 *********:******************:*************:******.**********

Zebrafish_RhoA   AGQEDYDRLRPLSYPDTDVILMCFSIDSPDSLENIPEKWTPEVKHFCPNAPIILVGNKKD 120
Zebrafish_Rhoab  AGQEDYDRLRPLSYPDTDVILMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKD 120
Fugu_RhoA        AGQEDYDRLRPLSYPDTDVILMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKD 120
Human_RHOA       AGQEDYDRLRPLSYPDTDVILMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKD 120
Human_RHOB       AGQEDYDRLRPLSYPDTDVILMCFSVDSPDSLENIPEKWVPEVKHFCPNVPIILVANKKD 120
Human_RHOC       AGQEDYDRLRPLSYPDTDVILMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKD 120
Mouse_RHOA       AGQEDYDRLRPLSYPDTDVILMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKD 120
Xenopus_RhoA     AGQEDYDRLRPLSYPDTDVILMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKD 120
                 *************************:*************.*********.*****.****

Zebrafish_RhoA   LRNDEHTRRELTKMKQEPVKAEEGRDMANRIGAFGYMECSAKTKDGVREVFEMATRAALQ 180
Zebrafish_Rhoab  LRNDEHTRRELTKMKQEPVKAEEGRDMANRIGAFGYMECSAKTKDGVREVFEMATRAALQ 180
Fugu_RhoA        LRNDEHTRRELAKMKQEPVKPEDGRDMANRISAFGYMECSAKTKDGVREVFEMATRAALQ 180
Human_RHOA       LRNDEHTRRELAKMKQEPVKPEEGRDMANRIGAFGYMECSAKTKDGVREVFEMATRAALQ 180
Human_RHOB       LRSDEHVRTELARMKQEPVRTDDGRAMAVRIQAYDYLECSAKTKEGVREVFETATRAALQ 180
Human_RHOC       LRQDEHTRRELAKMKQEPVRSEEGRDMANRISAFGYLECSAKTKEGVREVFEMATRAGLQ 180
Mouse_RHOA       LRNDEHTRRELAKMKQEPVKPEEGRDMANRIGAFGYMECSAKTKDGVREVFEMATRAALQ 180
Xenopus_RhoA     LRNDEHTRRELTKMKQEPVKPEEGRDMANRISAYGYMECSAKTKDGVREVFELATRAALQ 180
                 **.***.* **::******:.::** ** ** *:.*:*******:******* ****.**

Zebrafish_RhoA   ARRGKKS---NKCCLL 193
Zebrafish_Rhoab  ARRGKKS---NKCCLL 193
Fugu_RhoA        ARRGKKS---SKCAVL 193
Human_RHOA       ARRGKKK---SGCLVL 193
Human_RHOB       KRYGSQNGCINCCKVL 196
Human_RHOC       VRKNKRR---RGCPIL 193
Mouse_RHOA       ARRGKKK---SGCLIL 193
Xenopus_RhoA     ARRGKKK---PRCLLI 193
                  * ..:      * ::
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Figure 3.1 Amino acid sequence analyses of the Rho subfamily. (a) Alignment of zebrafish RhoA(AAO65961), zebrafish Rhoab (NP_997914.2), human RHOA (AAM21117), human RHOB (AAM21118), human RHOC (AAM21119), mouse RHOA (AAD52675), *Xenopus* RhoA (AAM47281), and Fugu RhoA (CA590877) proteins. "*" indicates the residues are identical in that column; ":" highlights the conserved substitution while "." shows the semi-conserved substitution. The colors represent the properties of residues. b) A phylogenetic tree of the zebrafish RhoA and other species generated from a ClustalW multiple sequence alignment.

3.1.2 Expression of *rhoA* in adult tissues and zebrafish embryogenesis

To understand the potential roles of zebrafish *rhoA* during vertebrate development, its spatial and temporal expression pattern was examined. Northern blot analysis using the specific 3'UTR probe shows that zebrafish *rhoA* gene expression is ubiquitously distributed in the adult fish, with a relative low level in the skin and muscle (Figure 3.2).

During embryonic development, *rhoA* expression was initially detected at the zygote period (1-cell stage, data not shown) by whole-mount in situ hybridization analyses. During the cleavage stage, the signals appeared to be distributed in the cytoplasm, with more intense expression in the centre of the cells in 80% of the embryos (Figure 3.3a-b). These signals coincided with nucleus as confirmed by DAPI staining

(Figure 3.3c-d). This expression pattern is similar to that of zebrafish zinc finger transcription factor Churchill (*chch*), and author has demonstrated that nucleus-localized expression is due to zygotic expression of *chch* prior to MBT [Londin *et al.* 2007]. In gastrula stage embryos, zebrafish *rhoA* was mainly expressed in the tissue undergoing extensive morphogenetic changes such as germ ring and dorsal midline. At 60% epiboly stage, zebrafish *rhoA* expression was enriched in the marginal zone and dorsal organizer (Figure 3.3e). The expression in the germ ring (Figure 3.3f) then persisted until it was completely closed. At the end of gastrulation, *rhoA* mRNA accumulated in both anterior head region (Figure 3.3g) and posterior tail bud (Figure 3.3h), and was intense in the dorsal midline (Figure 3.3g). In the brain, *rhoA* was initially expressed uniformly (Figure 3.3i-k). By the larval stage, the expression became intense in the medial and posterior of the tectum where the proliferation zones of midbrain is located [Wullimann *et al.* 2000], and also in the diencephalon as well as hindbrain (Figure 3.3m-o). It was reported that RhoA was present in the proliferation zones of the developing rat neocortex during neurogenesis, indicating that it is involved in neuron generation, migration and differentiation [Olenik *et al.* 1999]. The expression of *rhoA* in otic vesicle and retina was also detected (Figure 3.3n-q). Similar to our finding, RhoA mRNA was detected in chick retina suggesting that it was important for cell differentiation and the formation of synapses in the retina [Santos-Bredariol *et al.* 2002]. Besides the nervous system, zebrafish *rhoA* gene expression was elevated in the pharyngeal arches (Figure 3.3k), pronephric duct (Figure 3.3i-j), notochord (Figure 3.3i, k and n), heart (Figure 3.3k, n and p) and liver (Figure 3.3l, n and q). RhoA has been shown to regulate neural crest migration and cardiomyocyte proliferation [Wei *et al.* 2002], and is up-regulated in early

chick heart development [Kaarbo *et al.* 2003] as well as in rat kidney organogenesis [Bianchi *et al.* 2003].

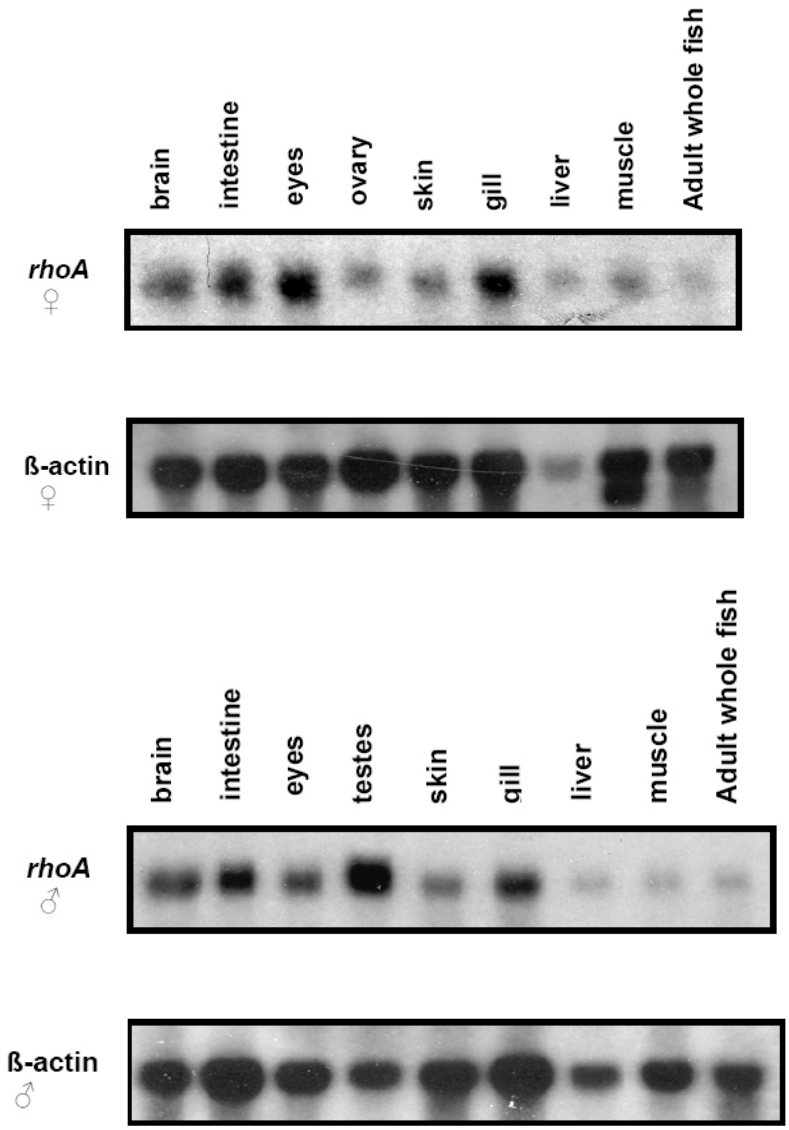

Figure 3.2 Expression of *rhoA* mRNA in adult zebrafish tissues. Northern blot analyses for *rhoA* in both female (♀) and male (♂) adult zebrafish tissues using the 3′ UTR specific probe prepared as described in Materials and Methods. β-actin was used as an internal control.

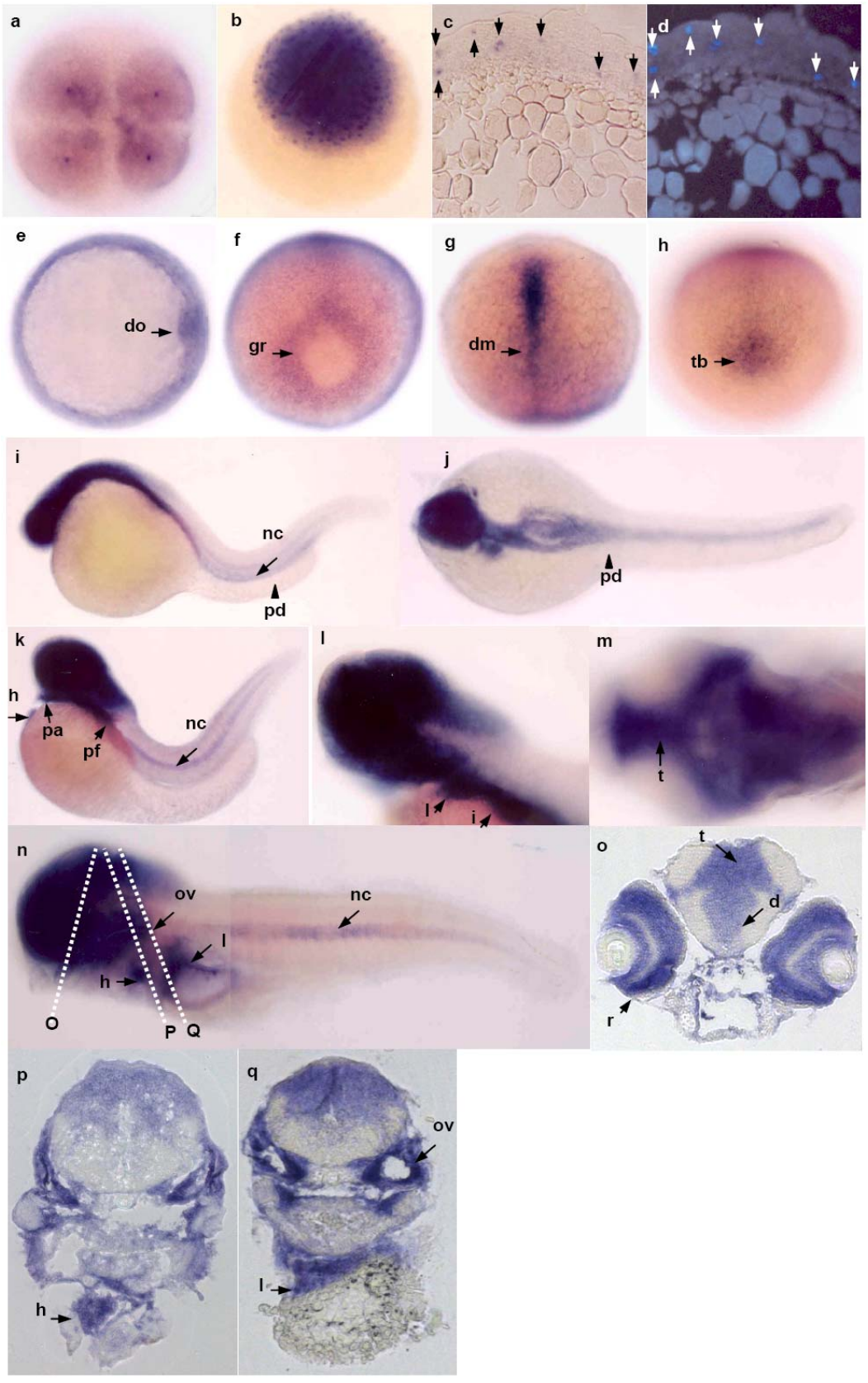
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Figure 3.3 In situ hybridization analyses for zebrafish *rhoA* expression in different stages of embryonic development. (a) 4-cell-stage embryo, animal view. (b) 128-cell-stage, animal view. (c-d) Longitudinal section of 128-cell-stage embryo, lateral view. (c) In situ hybridization; (d) DAPI staining; arrows indicating the overlapping expression of *rhoA* with nucleus staining. (e) Shield stage embryo, animal view with dorsal to the right. (f) 90% epiboly stage embryo, vegetal pole view with dorsal to the top. (g-h) Tail bud stage embryo. (g) Dorsal view, anterior to the top. (h) Vegetal pole view with dorsal to the top. (i) 24 hpf, lateral view with anterior to the left. (j) 48 hpf, dorsal view, without body, anterior to the left. (k) 48 hpf, lateral view with anterior to the left. (l) 72 hpf, lateral view with anterior to the left. (m) 96 hpf, dorsal view with anterior to the left. (n) 96 hpf, lateral view with anterior to the left. White lines indicate the location of section shown in (o), (p), (q) respectively. (o-q) Anterior cross sections of 96 hpf embryo with dorsal to the top. d, diencephalon; dm, dorsal midline; do, dorsal organizer; gr, germ ring; h, heart; i, intestine; l, liver; nc, notochord; ov, otic vesicle; pa, pharyngeal arches; pd, pronephric duct; pf, pectoral fin; r, retina; t, tectum; tb, tail bud; hpf, hours post-fertilization.

3.1.3 Interference with RhoA function disrupts convergence extension movements during gastrulation and tail formation

To elucidate the functions of *rhoA* during zebrafish embryonic development, morpholino (MO)-mediated gene knockdown was applied. Two non-overlapping morpholinos (*rhoA* MO1 and *rhoA* MO2) were designed against the zebrafish *rhoA* gene. Injection of either morpholino produced similar phenotypes (Figure 3.4 b-c, e-f, h-i, k-l), whereas the injection of standard control morpholino had no effects (data not shown). The reduction of endogenous RhoA protein after *rhoA* morpholino knockdown had been detected by western blot analysis (see later Figure 4.3a), indicating the specificity of MOs targeting to *rhoA* transcript. Since the two *rhoA* MOs showed similar phenotypes, *rhoA* MO1 was applied for subsequent experiments.

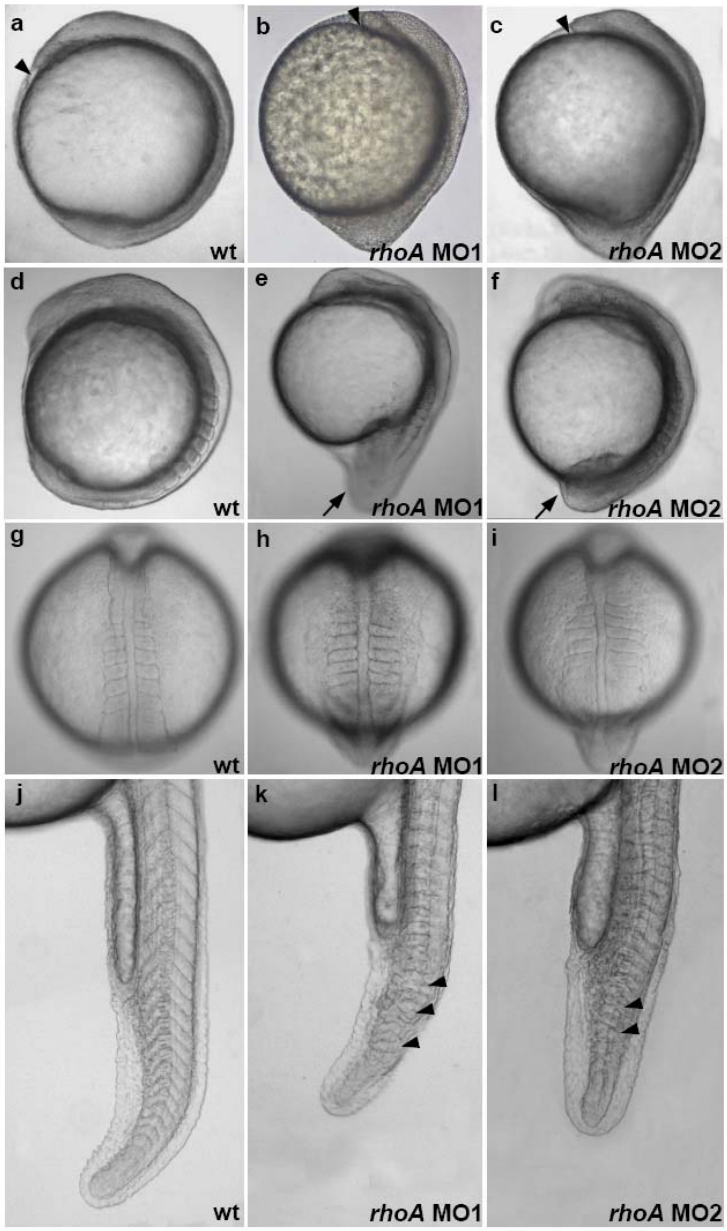


Figure 3.4 RhoA is required for zebrafish gastrulation and tail formation. (**a-l**) Phenotypic analysis of embryos injected with *rhoA* MOs. (**a-c**) 1-somite stage embryos, lateral view, dorsal to the right; arrowheads mark the anterior limit of the hypoblast layer. (**d-i**) 8 somites stage embryos. (**d-f**) Lateral view, dorsal to the right. Arrows point at the misprotruded tail (**e-f**). (**g-i**) dorsal view, animal pole to the top. (**j-l**) enlarged tail region of 24 hpf embryos; arrowheads indicate the malformed somites and the undulated notochord. Overall, *rhoA* morphants display a shorten anterior-posterior body axis, vegetally mispositioned head, detached tail from yolk, and malformed somites.

The *rhoA* morphants could be morphologically identified at the end of gastrulation. The embryos injected with a medium dose of *rhoA* MO1 (4 ng) displayed shorter anterior-posterior body axis, with reduced and posteriorly-positioned head (Figure 3.4b-c). During somitogenesis, the somites were compressed in the anterior-posterior axis and wider in the mediolateral axis (Figure 3.4h-i). The notochord was also shorter and broader, and mild undulation could be observed (Figure 3.4h-i). Similar defects were apparent in 1-day-old morphants. The notochord was more undulated and somites were not well-formed (Figure 3.4k-l). The morphological alterations in the axis of *rhoA* morphant indicate the defects in CE movements during gastrulation. These phenotypic defects can further be enhanced in a dose-dependent manner (Table 3.1).

Table 3.1 RhoA is required for zebrafish gastrulation and tail formation

			Phenotype at 13 *hpf*		
			Gastrulation and tail defects		
Injection	Amount injected per embryo	Total number of injected embryos	Wild type (%)	Severe (%)	Intermediate /mild (%)
control MO	8 (ng)	118	98.3	0	1.7
rhoA MO	2 (ng)	140	92.8	0	7.2
	4 (ng)	236	56.4	0.8	42.8
	8 (ng)	339	30.5	7	62.5
rescue	*rhoA* MO (8 ng)	139	28	7.2	64.8
	rhoA MO (8 ng) + *rhoA* mRNA (45 pg)	154	55.6	3.7	40.7*
	rhoA MO (4 ng)	121	67	0.8	32.2
	rhoA MO (4 ng) + *rhoA* mRNA (11.25 pg)	149	82.5	0	17.5*

Embryos were injected with control MO or the *rhoA* MO at the doses indicated, and observed for their defects in the presence or absence of co-injected *rhoA* mRNA for functional rescue. Embryos with their long tail detached from yolk, drastically reduced

head and anterio-posterior (AP) axis were scored as severe defects. Embryos with a reduced head and ventral movement of the tail bud along the yolk, shortened AP axis and an undulated notochord were scored as intermediate to mild defects. Differences from the control are statistically significant at $p<0.01$(*) for the rescue.

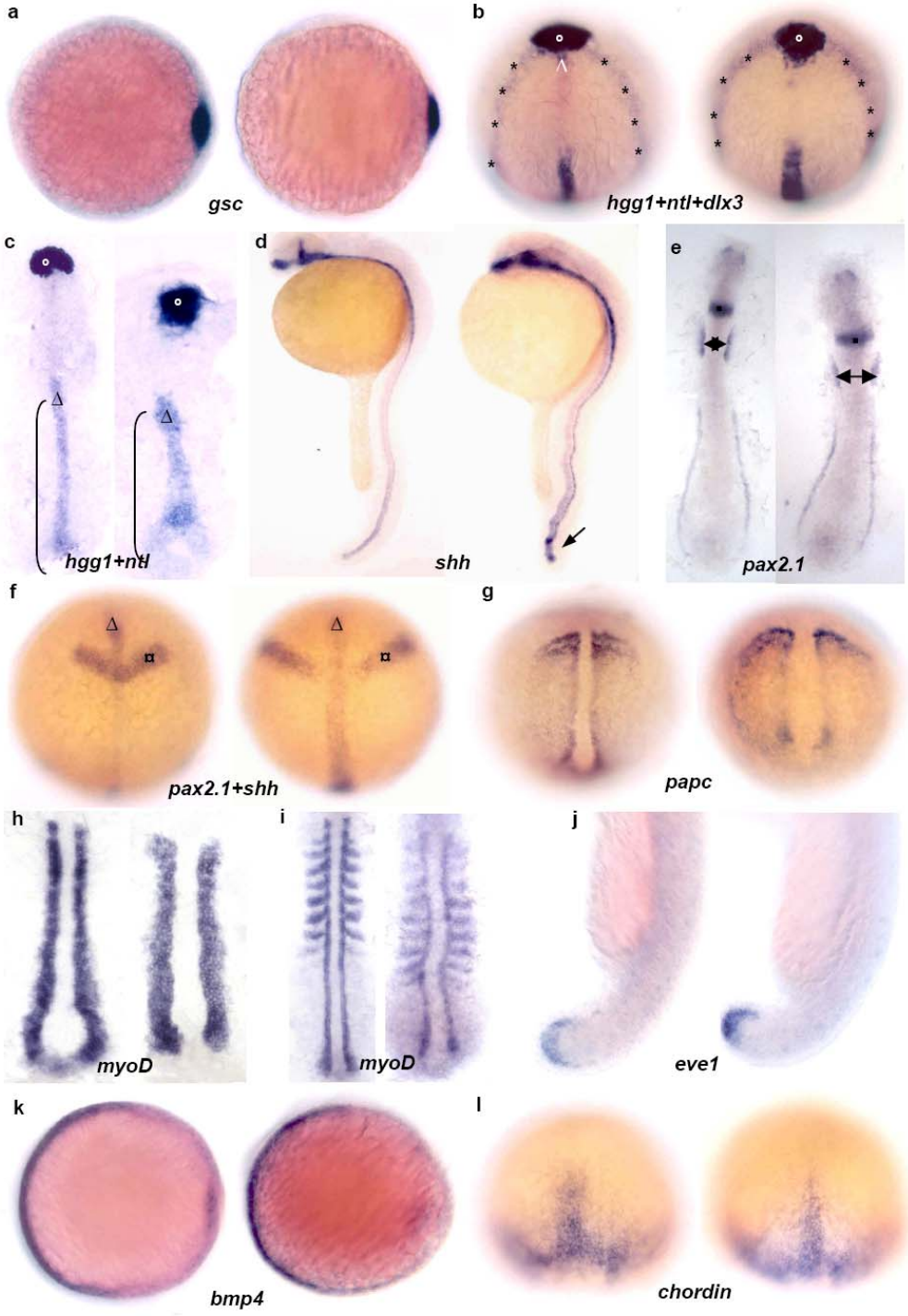
To further determinate the specificity of *rhoA* MO, a rescue experiment was performed. Because *rhoA* MO was designed against 5'UTR region of zebrafish rhoA, there is no complementry sequence between *rhoA* MO and *rhoA* mRNA. Coinjection of RNA encoding the full-length *rhoA* with *rhoA* MO could suppress the gastrulation and tail CE defects in a dose-dependent manner (Table 3.1). When 45 pg/embryo of synthetically capped *rhoA* mRNA was coinjected with 8 ng of *rhoA* MO, the magnitude of defected embryos significantly decreased and the severity of the phenotype reduced to intermediate or mild levels, further supporting that the morphant phenotyped indeed were the result of specific interference with the function of the endogenous RhoA.

In addition to the defects observed during gastrulation, the *rhoA* morphants also impaired in tail formation. Compared to the embryos injected with control MO (data not shown), the tail bud failed to move ventrally at the end of gastrulation, resulting in mis-protruded tail (Figure 3.4e-f) or the tail even being detached from the yolk sac during earlier development in some severely affected morphants (data not shown). Till 24 hpf, the yolk extension appeared shorter and thicker. The tail posterior to the yolk extension was shorter, and the malformed somites and undulated notochord were more severely affected than those at the trunk region (Figure 3.4k-l). These results suggest that a reduction in the functional RhoA activity disrupts both axial and paraxial mesoderm development during the tail formation. The low percentage of rescue could be due to the special sensitivity of embryos to the co-injection of rhoA mRNA with rhoA MO (data

not shown). It has been suggested that the early onset of translation of rescue mRNA in the early zygote may mess up the developmental process. Moreover, RhoA protein had been implicated to be maternally provided in zebrafish [Lai *et al.* 2005], which may compensate for the reduction of maternal mRNA translation caused by rhoA MO. Thus, to avoid the toxicity and over-expression phenotype caused by high expression of rhoA, sub-optimal doses of mRNA was applied which may not sufficient to restore normal development to all rhoA morphants. Alternatively, RhoA was ubiquitously expressed during early zebrafish embryogenesis [Zhu *et al.* 2006a]. Thus, the mosaic nature of the mRNA and MO injections reported by other researchers [McClintock *et al.* 2002; McWhorter *et al.* 2003] may also affect the efficiency of rescue.

3.1.4 Altered gene expression domains in *rhoA* morphants

To elucidate the underlying mechanism for the CE defects in the *rhoA* morphants, the expression of several specific gene markers that specify the axial, paraxial mesoderm and neuroectoderm in trunk and tail were examined. At the onset of gastrulation, the expression of the *goosecoid (gsc)* gene (Figure 3.5a), which marks the presumptive dorsal mesoderm [Stachel *et al.* 1993], appeared normal at the shield stage, indicating that *rhoA* knockdown did not prevent mesoderm induction or differentiation. At the end of gastrulation, the prechordal plate, marked by *hatching gland 1* (*hgg1*) expression (Figure 3.5b), was positioned slightly posteriorly with respect to the *dlx3* expression in the anterior edge of the neural plate. Thus, it appears that the most anterior axial mesoderm is least affected.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gsc
b
hgg1+ntl+dlx3
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hgg1+ntl
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pax2.1+shh
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papc
h
myoD
i
myoD
j
eve1
k
bmp4
l
chordin

Figure 3.5 Expression of marker genes in *rhoA* morphants. Wild type embryos and embryos injected with *rhoA* morpholino were analyzed for the expression domains of the marker genes by in situ hybridization. In each section (a-l), wild type is shown on the left and *rhoA* morphants on the right. Please refer to the text for detailed description. (b) *gsc*, shield stage, animal view, dorsal to the right; (b) *hgg1*, *ntl* and *dlx3*, tail bud stage, dorsal view: neural plate (*) is wider and prechordal plate (○) is slightly posteriorly located. (c) *hgg1* and *ntl*, tail bud stage, dorsal view on spread embryo, anterior to the top: notochord (Δ) is broader and shorter. (d) *shh*, 24 hpf, lateral view, dorsal to the right: notochord is wider and undulated (marked by arrow). (e) *pax2.1*, 8-somite stage, dorsal view on spread embryo, anterior to the top: mid-hind brain boundary (▪) and the distance between otic placode is wider (shown by arrow). (f) *pax2.1* and *shh*, tail bud stage, dorsal view, anterior to the top: *rhoA* morphant displays wider neural plate (¤) and notochord (Δ). (g) *papc*, 2-somite stage, dorsal view, anterior to the top: the posterior paraxial mesoderm expands laterally. (h, i) *myoD*, bud stage (h) and 9-somite stage (i), dorsal view on spread embryo, anterior to the top: somites are a little compressed and laterally expanded. (j) *eve1*, 20 hpf, lateral view, dorsal to the right: *eve1* expression is normal in the tail bud. (k) *bmp4*, 70% epiboly, animal view, dorsal to the right: the expression domain of *bmp4* in the ventral marginal zone was unaffected. (l) *chordin*, 70% epiboly stage, dorsal view: the expression domain of *chordin* remains unchanged.

In contrast to the prechordal plate, the anterior-posterior extension and medio-lateral convergence of the axial mesoderm was reduced in *rhoA* morphants, leading to shorter and broader notochord at the end of gastrulation, as indicated by the expression of *no tail (ntl)* [Schulte-Merker *et al.* 1994] (Figure 3.5c) and *sonic hedgehog* (*shh)* [[Krauss *et al.* 1993] (Figure 3.5f). At 24 hpf, the undulated notochord was obvious and appeared to be more affected in the tail region posterior to the yolk extension than in the trunk part. Sometimes it was even folded (Figure 3.5d). This shortened and broadened midline in *rhoA* morphant supports that the CE defect was associated with dorsal mesoderm.

In the paraxial mesoderm, two strips of adaxial cells were mediolaterally expanded and anterior-posteriorly shortened, as revealed by *myoD* expression [Weinberg *et al.* 1996] at the end of gastrulation (Figure 3.5h). The expression domain of *myoD* appeared to be more extended in the posterior somatic mesoderm and compressed in anterior-posterior axis during somitogenesis (Figure 3.5i). The similar broader and shorter posterior paraxial mesoderm was shown by *paraxial protocadherin* (*papc)* [Yamamoto *et al.* 1998] staining at the early somites stage (Figure 3.5g). Thus, the CE movements in *rhoA* morphant were also inhibited in the non-axial mesoderm.

In neuroectoderm, the laterally expanded expression domain of *distal-less3* (*dlx3)* [Akimenko et al. 1994] (Figure 3.5b) and *pax2.1* [Krauss *et al.* 1991] (Figure 3.5e-f) revealed broader neural plate. Compared to the control, the expression domain of *pax2.1* in the mid-hindbrain boundary (Figure 3.5f) and otic placode in *rhoA* morphants (Figure 3.5e), extended obviously but without changes in the cell fate. In addition, the expression pattern of the ventral ectoderm marker, *bone morphogenetic protein4* (*bmp4)* [Fainsod *et al.* 1994] (Figure 3.5k), the dorsal mesoderm marker, *chordin* [Sasai *et al.* 1994] (Figure 3.5l), as well as the tail bud marker, *eve1* [Joly *et al.* 1993] (Figure 3.5j) were all unaffected. These results indicate that lack of RhoA function does not affect dorsal-ventral patterning and tail bud differentiation.

3.1.5 RhoA is required for both Wnt5 and Wnt11 signaling to induce gastrulation movement

In vertebrates, the Fz/Dsh PCP pathway is essential for cell polarity and movement during gastrulation [Sokol 2000]. In *Xenopus,* RhoA, together with another member of Rho small GTPases, Rac, mediates Wnt11/Frz signaling that regulates

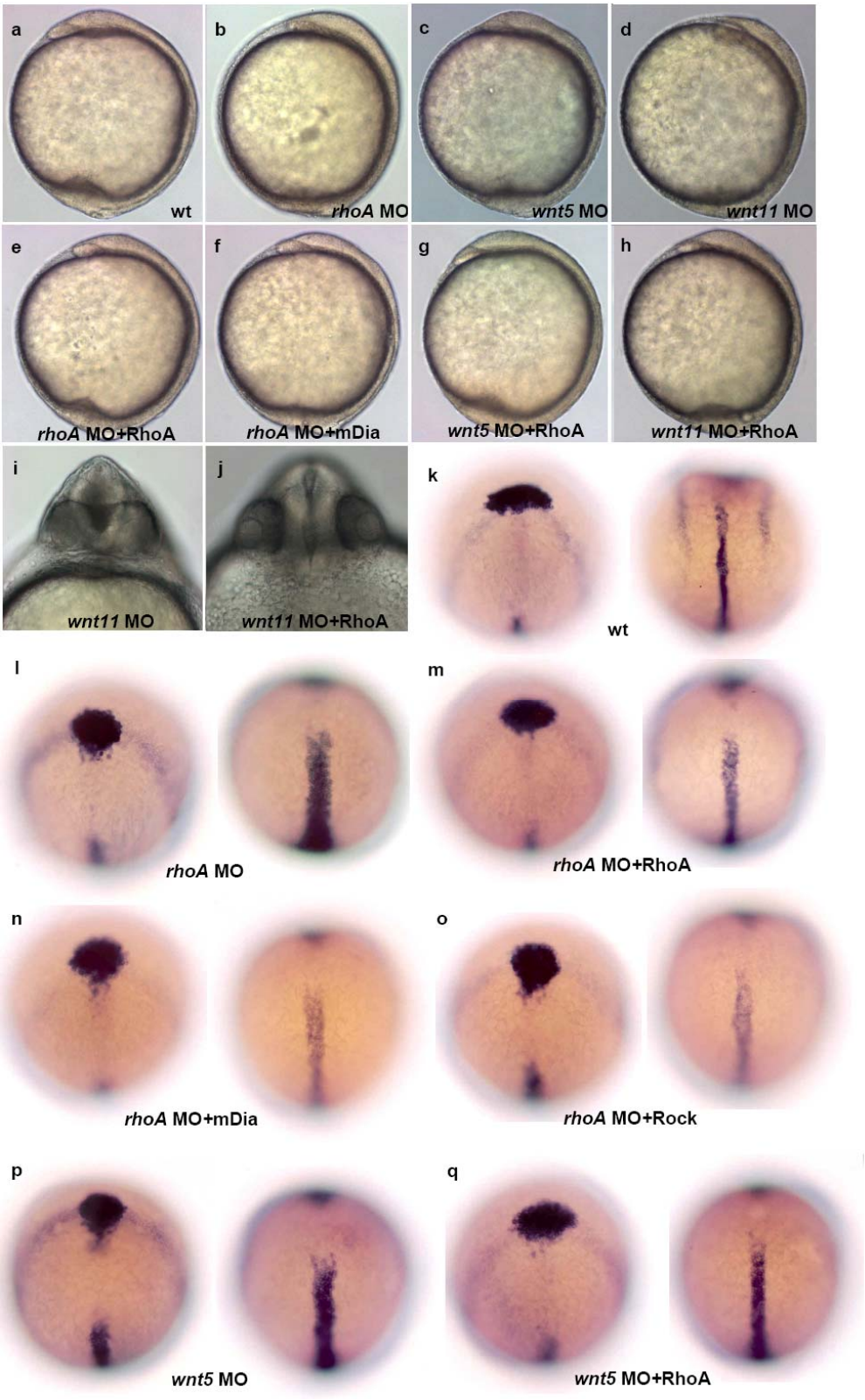
gastrulation movements [Habas *et al.* 2003] whereas the RhoA effector Rho kinase 2 acts downstream of Wnt11 to mediate cell polarity and CE movement in the zebrafish [Marlow *et al.* 2002]. Since Rho kinase is a more potent effector for RhoC than RhoA or RhoB [Wheeler *et al.* 2004] and that these findings are derived from ectopic expression of constitutive active or dominant negative mutants of Rho GTPase [Barrett *et al.* 1997; Tahinci *et al.* 2003] or their effectors [Marlow *et al.* 2002], any non-specific functional interference to closely-related signaling proteins could not be completely ruled out. It remains to be seen which of the Rho GTPases is/are indeed involved during the CE movements in the zebrafish gastrula. Furthermore, *ppt/wnt5* whose function partially overlaps with *slb/wnt11*, is also required for cell elongation and CE movements in the posterior mesendoderm during late gastrulation [Kilian *et al.* 2003]. Therefore, we hypothesize that RhoA could function downstream of both Wnt5 and Wnt11 for the CE movement during zebrafish gastrulation. Furthermore, we wished to establish whether effectors other than Rho kinase could also serve as downstream determinant of RhoA in eliciting CE movements. To address this issue, functional rescues of CE defects in *wnt* mutants were performed using *rhoA* mRNA. Different concentrations of *wnt11* and *wnt5* morpholinos were titrated to reach an optimal level that could best phenocopy the *slb*-specific gastrulation and eye phenotype and *ppt*-posterior gastrulation defects, which are indicative of their respective CE defects [Rauch *et al.* 1997; Heisenberg *et al.* 2000]. Same amount of *rhoA* mRNA used in the earlier *rhoA* morphant rescue experiment was then applied to the *ppt/wnt5* and *slb/wnt11* knockdowns. As determined by in situ hybridization and morphological criteria, RhoA could significantly ($p<0.01$) suppress both the reduction in anterior–posterior extension of the prechordal plate (Figure 3.6t-u)

and the cyclopia in *wnt11* morphant (Figure 3.6i-j; Table 3.2). For the *wnt5* rescue, nearly half of the embryos restored the normal body length (Figure 3.6c and g; Table 3.2), while the notochord and neural plate appeared normal (Figure 3.6p-q) upon injection of RNA encoding full-length *rhoA*. These results strongly suggest that RhoA is required for both Wnt5 and Wnt11 signaling in governing CE movements in the zebrafish embryos.

Table 3.2 RhoA, mDia and Rock suppress zebrafish gastrulation defects caused by *rhoA*, *wnt5* and *wnt11* morpholinos

Injection	Total number of embryos injected	CE Mutant embryos (%)	Standard deviation
wnt11 MO (4 ng)	129	39.5	3.9
wnt11 MO (4 ng) + *rhoA* mRNA (11.25 pg)	151	19.2*	5.2
wnt5 MO (8 ng)	217	71.9	6.6
wnt5 MO (8 ng) + *rhoA* mRNA (11.25 pg)	145	39.3*	2.8
rhoA MO (6 ng)	328	58.8	1.9
rhoA MO (6 ng) + pEG-mDia (120 pg)	308	43.5**	3
rhoA MO (6 ng) + pCAG-*myc*-p160Rock (120 pg)	281	44.1**	5.4
rhoA MO (6 ng) + pEG-mDia (120 pg) + pCAG-*myc*-p160Rock (120 pg)	250	34.8*	3.2
wnt11 MO (4 ng)	335	54.3	1.5
wnt11 MO (4 ng) + pEG-mDia (120 pg)	199	38.2**	4.9
wnt11 MO (4 ng) + pCAG-*myc*-p160Rock (120 pg)	183	41.5**	5.8
wnt11 MO (4 ng) + pEG-mDia (120 pg) + pCAG-*myc*-p160Rock (120 pg)	253	31.6*	4.4
wnt5 MO (8 ng)	204	65.2	2.7
wnt5 MO (8 ng) + pEG-mDia (120 pg)	168	51.2**	2.8
wnt5 MO (8 ng) + pCAG-*myc*-p160Rock (120 pg)	132	49.2**	3.9
wnt5 MO (8 ng) + pEG-mDia (120 pg) + pCAG-*myc*-p160Rock (120 pg)	173	39.9*	2.7

Morpholino corresponding to RhoA, Wnt5 or Wnt11 were injected into embryos as described in Materials and Methods. Embryos with reduced anterio-posterior (AP) axis and broader medial-lateral axis at the end of gastrulation were scored as defected embryos. Each experiment was repeated at least three times and paired Student's *t*-test were performed. Differences from the controls are statistically significant at $p < 0.01$(*) for RhoA rescue and combined mDia and Rock rescue group, or at $p < 0.05$ (**) for the respective single rescue group from mDia or Rock.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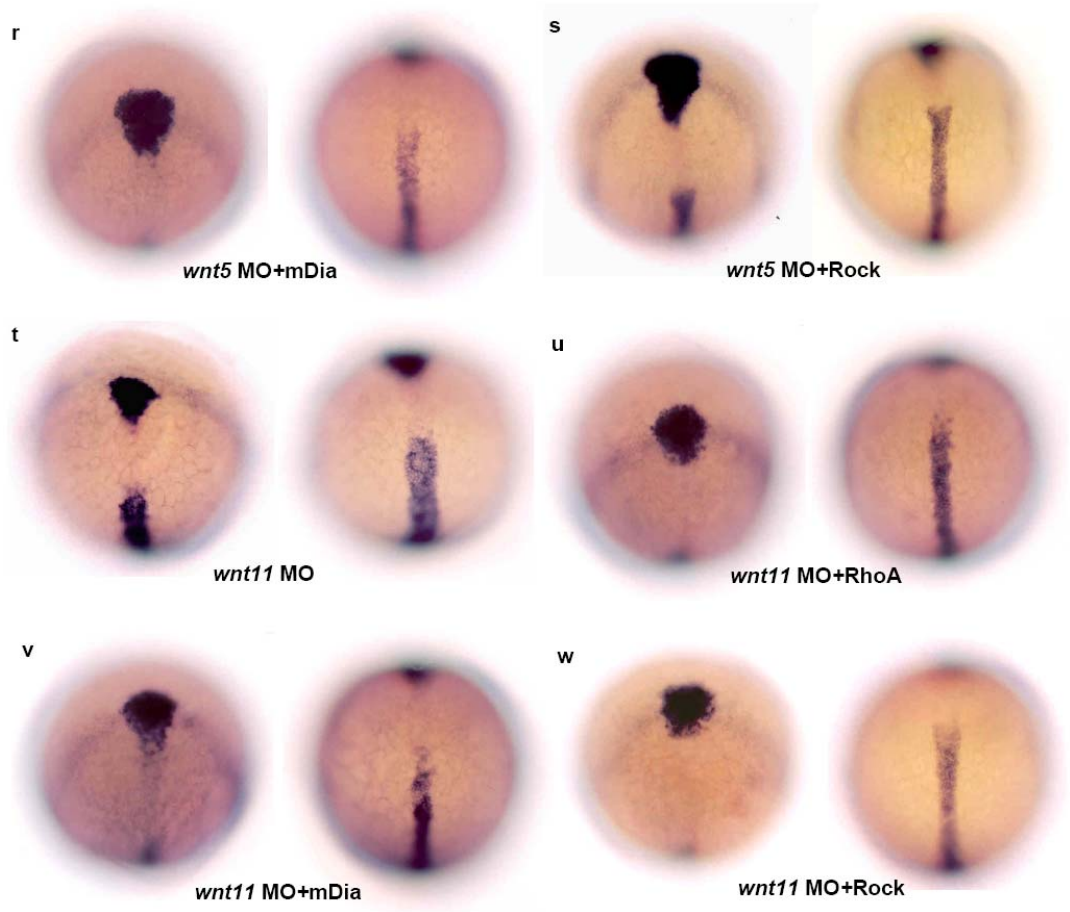


Figure 3.6 RhoA and mDia suppress *wnt5* and *wnt11* morphants. (a-h) Lateral view and (k-m) dorsal view of tail bud-stage wt, *rhoA*, *wnt5*, *wnt11* morphants and rescued embryos upon expression of zebrafish RhoA, human mDia, or human Rock. (i-j) ventral view of 24 hpf *wnt11* morphant (Cyclops) and RhoA rescued embryo. At the end of gastrulation, the short and broad notochord (*ntl*) in *rhoA* (b, l), *wnt5* (c, p) and *wnt11* (d,t) morphants was suppressed by the coinjection of full-length *rhoA* RNA (e, g, h, m, q and u), mDia plasmid (f, n, r and v), or Rock plasmid (o, s and w). Defects of *wnt11* morphant in prechordal plate (*hgg1*) posterior positioned to neuroplate (*dlx3*) (t) and cyclopia (i) were also rescued by RhoA (u and j), mDia (v) or Rock (w).

3.1.6 Rho kinase and Dia function downstream of RhoA and Wnt in controlling CE movement

RhoA plays an important role in cell movements by regulating reorganization of the actin cytoskeleton. This action is cooperatively mediated by its two downstream Rho effectors, Rho kinase and mDia [Watanabe *et al.* 1999]. However, there is no report showing the involvement of mDia in the CE movement. To investigate this, a series of further rescue experiments were performed by the expression of the human Rho kinase or/and mDia in the embryos coinjected with either *rhoA* or *wnt* morpholinos. Compared with the embryos injected with *rhoA* MO alone, embryos co-injected with pCMV-mDia and *rhoA* MO exhibited a marked reduction in the number and extents of gastrulation defect ($p<0.05$) (Figure 3.6f and n; Table 3.2). Consistently, mDia also effectively suppressed the CE defects in the *wnt5* and *wnt11* morphants (Figure 3.6r and v; Table 3.2), further supporting that mDia is indeed involved in the regulation of CE movements by acting downstream of RhoA and Wnt signaling. Similar suppression of CE defects in the *rhoA* and *wnt* morphants was also observed when a human Rho kinase was expressed in the embryos (Figure 3.6o, s and w). RhoA thus acts downstream of the noncanonical Wnt signaling to control CE movement via at least two of its effectors, Rho kinase and Dia.

3.2 Discussion

3.2.1 RhoA function is required for convergence extension movements during gastrulation and tail formation

The spatial and temporal expression profiles of *rhoA* provide the first hint for its function in gastrulation. At the onset of gastrulation, *rhoA* is highly expressed in both dorsal and ventral marginal zone and dorsal organizer where dorsal mesoderm starts to internalize. Then during gastrulation, *rhoA* maintains high expression levels in marginal cell layer, which is the leading edge of active cell movement, and its expression in dorsal midline is more intense when the dorsal anterior-posterior extension occurs. At the end of gastrulation, its expression is enriched in the head and tail bud region, suggesting that *rhoA* is also important for head and tail formation, similar to the findings in *Xenopus* where RhoA induces the formation of head structures [Wunnenberg-Stapleton *et al.* 1999]. Taken together, *rhoA* is expressed throughout the gastrulation, and is more highly expressed in cells undergoing extensive morphogenetic changes and movements.

Moreover, *rhoA* morphants display embryonic defects similar to those when the convergent extension is disrupted. Reduced body axis, broader and compressed somites, shorter, broader and undulated notochord in *rhoA* morphant are reminiscent of the phenotype in *trilobite* (*tri*) mutants [Hammerschmidt *et al.* 1996]. Furthermore, the gastrulation defects appear to arise from morphogenetic problems and are not due to earlier failures in the mesoderm induction, since gene specific to axial mesoderm, eg. *goosecoid (gsc)* is still expressed. The mesoderm specification is also unaffected, because notochord and muscle are still differentiated as shown by *ntl* and *myoD* staining. Furthermore, *rhoA* morphants can be rescued in a dose-dependent manner by injection of

mRNA encoding full-length *rhoA*, indicating CE defect arose due to the loss of RhoA function. Consistent with our findings in zebrafish, RhoA was reported to control cell motility in convergent extension of axial mesoderm in *Xenopus* [Tahinci *et al.* 2003]. Furthermore, in *Drosophila*, inhibition of RhoA signaling, by injection of dominant negative mutant of RhoA, disrupted gastrulation [Barrett *et al.* 1997]. Beside this, zebrafish Rho kinase was also shown to mediate cell polarity and exert effect on convergence and extension movements [Marlow *et al.* 2002]. Therefore, the function of RhoA in gastrulation dynamics is conserved in both invertebrate and vertebrate.

RhoA is not only required for convergent extension movements during gastrulation but also important for tail formation. Previous studies have shown that both the continuation of convergent extension movement initiated during gastrulation and novel tailbud-specific movement contribute to the tail formation [Kanki *et al.* 1997; Marlow *et al.* 2004]. The cells originating from dorsal gastrula regions occupy the anterior tailbud, and continue gastrulation-like convergence extension resulting in the elongation of axial mesoderm of the tail, whereas the ventrally originating cells constitute the posterior half and undergo subduction and then lateral divergence/CE movement, giving rise to the paraxial mesoderm of tail [Kanki *et al.* 1997]. In *rhoA* morphants, the obvious sign of tail defect can be first identified at 2-somites stage. After gastrulation, the tail bud fails to move ventrally on the yolk sac. Then till 24 hpf, the tail including yolk extension and the region posterior to that becomes shorter concurrent with the shorter body axis. These phenotypes are reminiscent of the tail mutants, *pipetail* (*ppt*) and *knypek (kyn)* [Hammerschmidt *et al.* 1996; Rauch *et al.* 1997]. The tail elongation defect in *ppt* mutants is neither due to the failure to specify or maintain posterior tissues,

nor decreased cell proliferation or increased cell death, but rather an impaired gastrulation-like convergence and extension movements and cell movements within the posterior tailbud [Hammerschmidt *et al.* 1996]. Beside those phenotypes similar to *ppt* mutants, *rhoA* morphants display more severe defects in the region posterior to the yolk extension than in trunk region: the notochord is more undulated and somites are malformed in the most severe embryos. Based on the expression of *rhoA* at both dorsal and ventral marginal zone at gastrula, we hypothesize that the inhibition of the dorsal expression of *rhoA* might cause less axial extension movement which results in undulated notochord in tail, whereas disruption of ventral *rhoA* expression could block the posterior tail-specific movement leading to abnormal development of somites. In addition to the spatial-temporal expression of *rhoA*, its upstream regulators or downstream effectors might also have differential distribution in gastrula embryo leading to the distinct signaling pathways in the regulation of tail and trunk development. In our study, we found that RhoA acts downstream of Wnt11 and Wnt5 in regulating gastrulation movement and tail formation. Wnt11 is known to be distributed and functions more anteriorly whereas Wnt5 acts more posteriorly [Kilian *et al.* 2003]. It has also been identified that Daam1 mediates RhoA activation by Wnt11 through a RhoGEF-dependent manner during *Xenopus* gastrulation [Habas *et al.* 2001]. However, it remains unclear whether Daam1 or other regulators of RhoA exist in Wnt5 noncanonical pathway that could lead to RhoA activation in the posterior part of embryos.

3.2.2 Wnt5 and Wnt11 requires RhoA in regulating CE movement

RhoA, as a key regulator of actin cytoskeleton, mediates the establishment of planar cell polarity of ommatidia and wing hairs in *Drosophila* via PCP pathway [Fanto

et al. 2000]. In vertebrate, noncanonical Wnt signaling pathway similar to PCP pathway in *Drosophila* plays important roles in regulating convergence and extension movement during gastrulation. Several zebrafish mutants were found to exhibit reduced convergent extension movements without affecting cell fates, including two noncanonical Wnt ligand genes, *wnt11* and *wnt5*. The *silberblick* (*slb*) /*wnt11* mutant shows more anterior CE defects: shortened and broadened body axis at the end of gastrulation and a slight fusion of the eyes (Cyclops) at later developmental stages. In contrast, *pipetail* (*ppt*)/*wnt5* functions in posterior regions exhibiting a shortened body axis and compressed tail while the position of the eyes is only mildly affected [Rauch *et al.* 1997; Heisenberg *et al.* 2000]. Although *wnt11* and *wnt5* function at different regions, they exhibit partially overlapping functions in regulating CE movements in lateral domains of the gastrula. The redundant function of Wnt11 and Wnt5 might be due to the sharing of same cellular component(s) in response to the signals. Supporting this, we found that *rhoA* was expressed in the entire germ ring during gastrulation, and coinjection of RNA encoding full-length *rhoA* with *wnt11* MO or *wnt5* MO indeed could suppress the phenotypic defects due to the loss of Wnt11 and Wnt5, respectively. This result strongly indicates that RhoA is the downstream mediator for both Wnt11 and Wnt5.

3.2.3 Rock and Dia mediate Wnt-RhoA signaling in gastrulation and tail formation.

The function of RhoA in regulating *Drosophila* planar cell polarity and vertebrate gastrulation has been shown to require Rho kinase as one of its effectors [Winter *et al.* 2001]. In zebrafish, Rho kinase 2 acts downstream of Wnt11 to regulate cell polarity as well as the convergence and extension movements during gastrulation [Marlow *et al.* 2002]. Moreover, Matsui et al (2005) has recently used the dominant negative mutant of

RhoA to show that it also acts downstream of Wnt4a, Wnt11 and Wnt11-related to primarily regulate the convergence of heart primordia during zebrafish development [Matsui *et al.* 2005]. However, it is not clear whether it would involve Rho kinase or/and Dia as observed in our current studies for the CE movements of mesoderm during gastrulation. In our study, we showed that expression of the human mDia effectively suppressed the CE defects due to RhoA knockdown, confirming that mDia activity is important for zebrafish gastrulation and tail formation. The temporal and localized stabilization of microtubules and actin plays crucial roles during many morphogenetic events, including cell migration, muscle development, neurite outgrowth, and epithelial polarization [Bulinski *et al.* 1991; Gundersen *et al.* 1999]. mDia mediates RhoA-induced microtubule formation [Palazzo *et al.* 2001] and is involved in the regulation of microtubule dynamics by stabilizing the microtubule at the leading edge of migrating fibroblast cells [Palazzo *et al.* 2004]. In addition, mDia can induce actin polymerization [Watanabe *et al.* 1997] and cooperate with Rho kinase for alignment of actin bundles to form stress fiber and focal adhesion [Watanabe *et al.* 1999] that provides the contractile forces for cell movement. Our results therefore are consistent with the view that mDia, like Rho kinase, is also a key component of the RhoA-induced cell polarization and migration during zebrafish gastrulation and tail formation (Figure 3.7).

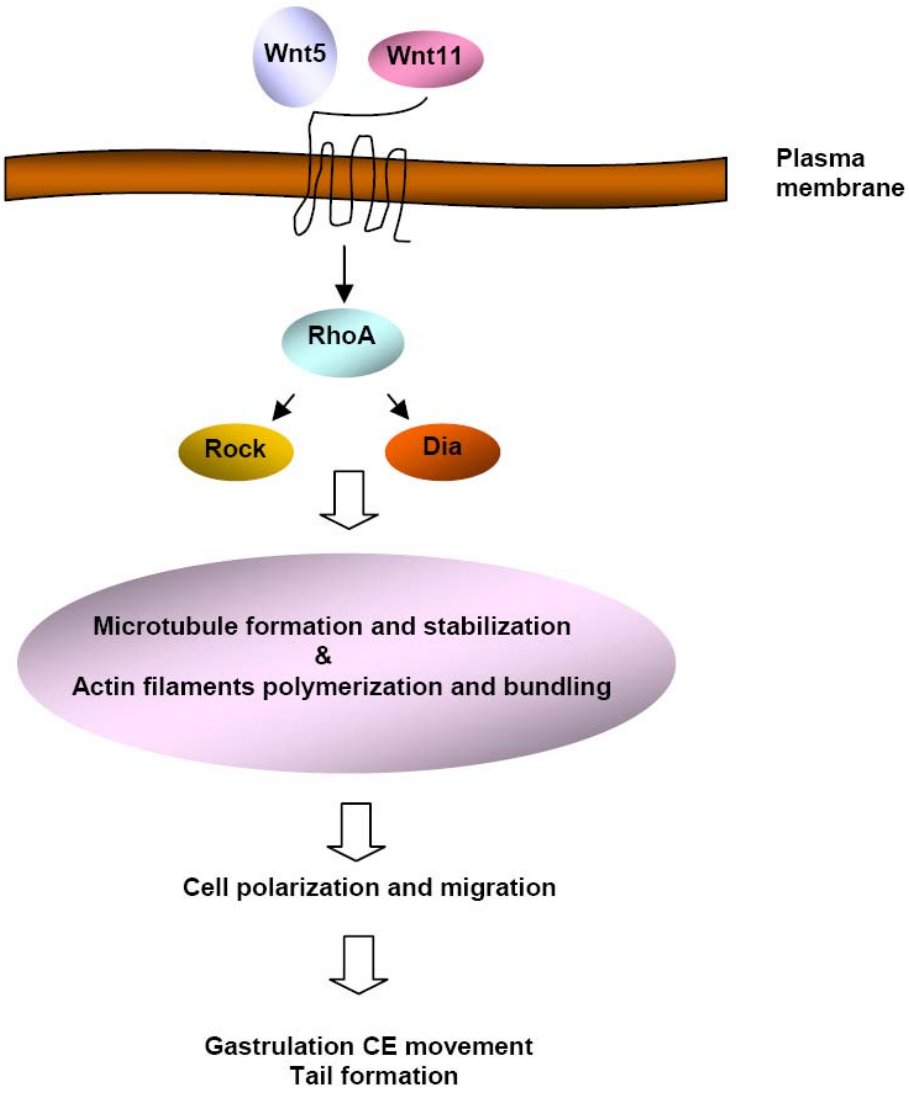


Figure 3.7 Wnt/RhoA signaling pathway regulates CE movement in zebrafish embryos via Rho kinase and Dia. During zebrafish gastrulation, RhoA can be activated by both Wnt5 and Wnt11 signaling. The activation of RhoA leads to actin polymerization and microtubule dynamics via cooperative effects of Dia and Rho kinase, Rock. Consequently, the RhoA-induced cell polarization and migration could lead to convergence and extension movement during the gastrulation and tail formation in the zebrafish embryos.

3.3 Conclusion

In summary, our morpholino-based functional knockdown of noncanonical Wnt and RhoA has established their direct functional interaction necessary for regulating cell dynamics *in vivo*. In this regard, at least Rho kinase and Dia represent two key determinants in mediating their downstream effects in the CE movements. Given the efficacy in functional knockdown by morpholinos and relative ease of embryonic studies, our results implicate the suitability of the zebrafish as a vertebrate model to further our understanding on the roles of various classes of small GTPases signaling in the control of cellular and developmental dynamics.

Chapter 4 RhoA prevents apoptosis during zebrafish embryogenesis through activation of Mek/Erk pathway

Extending our finding that RhoA links Wnt signaling to the gastrulation movements in the zebrafish, we further explore the in vivo signaling mechanism of RhoA during post-gastrulation periods of embryogenesis. In this chapter, we report that knockdown of RhoA by its specific anti-sense MOs causes marked increase in apoptosis during embryogenesis, leading to overall reduction in body size and length as well as severe shrinkage of brain. The knockdown of RhoA is also closely associated with reduced phosphorylation status of the growth-promoting kinase, Erk and a lack of expression of the key anti-apoptotic protein bcl-2. Using a combination of gene suppression and functional rescue experiments together with specific pharmacological inhibitors for Mek and Bcl-2, we show that RhoA prevents Bcl-2-dependent intrinsic apoptosis during zebrafish embryogenesis by activation of Mek/Erk signaling pathway.

4.1 Results

4.1.1 RhoA knockdown results in reduced body size and shortened body length in zebrafish embryos

To study the role of RhoA in zebrafish embryogenesis beyond gastrulation, the two non-overlapping *rhoA* MOs (*rhoA* MO1 and *rhoA* MO2) which have been demonstrated to knockdown RhoA successfully in my previous study and a 5-bp mismatch MO of *rhoA* MO1 were applied. During the whole embryogenesis, the standard control MO- or 5-mismatch MO-injected embryos developed normally (Figure 4.1a-b, f-g,

i, k-l and p-q). In contrast, embryos injected with *rhoA* MO1 and *rhoA* MO2 displayed convergence and extension defects at the end of gastrulation (Figure 4.1c-d) as we reported previously [Zhu *et al.* 2006b]. During early somitogenesis, the apparent opaque region was observed in the head of *rhoA* morphants (Figure 4.1h), and cell corpuses were detected within or at the boundary of deformed somites (Figure 4.1j). By mid-segmentation stage, the opaque regions were found throughout the morphants, but most were detected in head and tail ("opaque" phenotype) (Figure 4.1m-n). Till pharyngula stage, *rhoA* morphants exhibited shrinkage in the whole body, with severely reduced size in head and shortened body length including yolk extension and tail posterior to the yolk extension (Figure 4.1r-s). Most of the severely affected *rhoA* morphants died within 4 days. Some of the mildly defected embryos could survive longer, but they never reached the similar body size and length as the control morphants (data not shown). The similar phenotype induced by two non-overlapping *rhoA* MOs suggests that the "opaque" phenotype and overall reduction of body size and body length in *rhoA* morphants could be resulted from specific knockdown of RhoA and is unlikely due to the non-specificity of MOs applied.

To further rule out that such morphants are secondary effect of cell movement defects during gastrulation, one splice MO (E3I3 MO) targeting the 3rd exon/intron boundary of *rhoA* was designed as described by Morcos [Morcos 2007]. Compared to *rhoA* MO1&2, E3I3 MO did not cause cell movement defects at the end of gastrulation (Figure 4.1e), but it induced the "opaque" phenotype in *rhoA* morphants after gastrulation (Figure 4.1o and t). Hence, the result supports that the maternal *rhoA* contributes to the

control of gastrulation movement more than zygotic *rhoA*, and the opacity in *rhoA* morphants is independent of the cell movement defects during gastrulation.

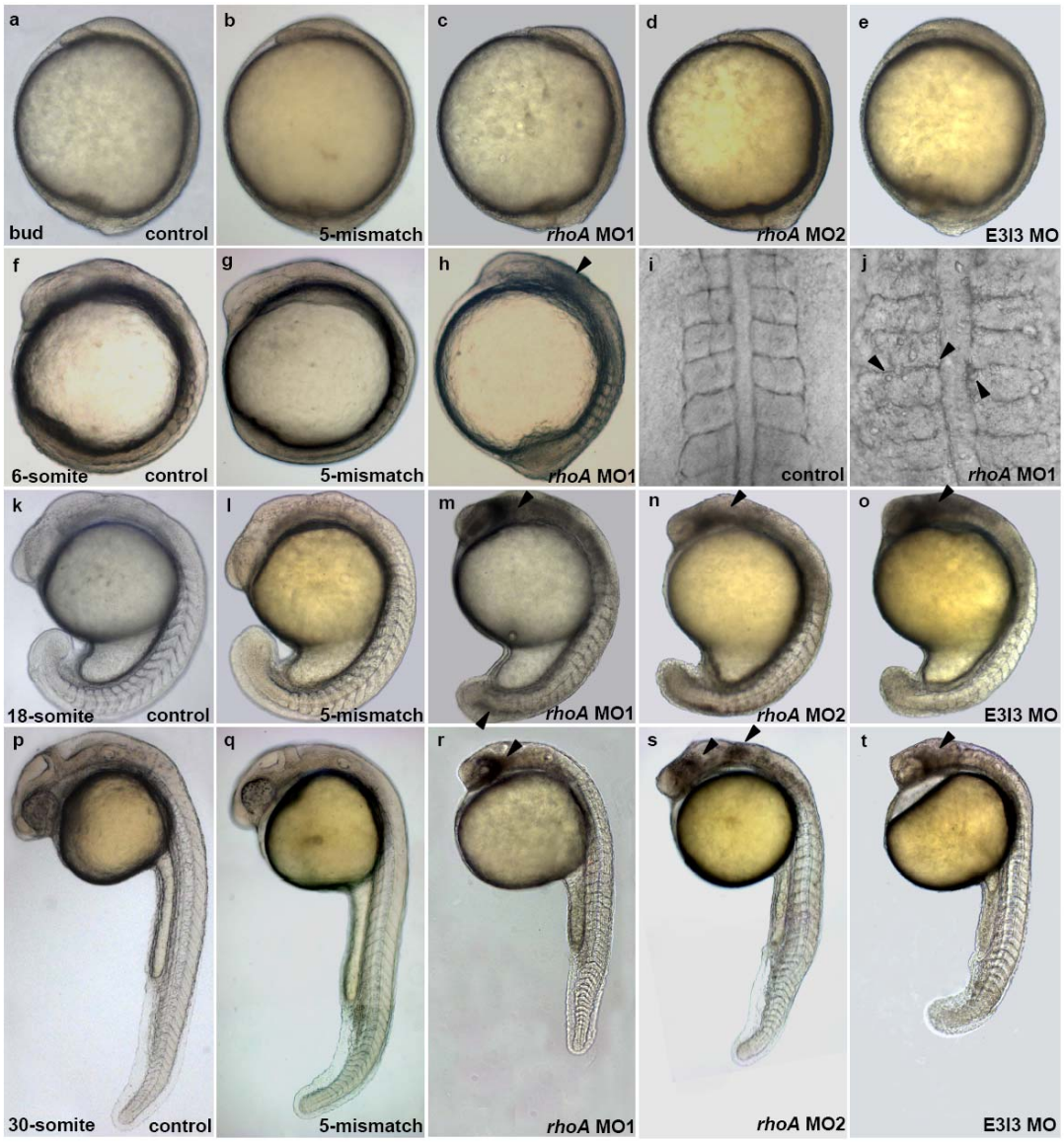


Figure 4.1 RhoA knockdown causes reduced body size and body length in zebrafish embryos. (a-t) Phenotypic analysis of embryos injected with control MO (control), 5-bp mismatch MO and *rhoA* MOs. (a-h and k-t) lateral view, dorsal to the right, (i and j) dorsal view, animal pole to the top. Embryos at the same stage are shown on the same row, and the developmental stages are indicated in the first column. Arrow heads highlight the opaque regions in head and tail (h, m-o and r-t) and the cell corpses within or around somites in *rhoA* morphants (j).

4.1.2 RhoA knockdown induces apoptosis during zebrafish embryogenesis

It is reported that the opaque regions in the developing zebrafish embryos contain apparent cell death and could be a consequence of apoptosis in some cases [Langheinrich *et al.* 2002; Liu *et al.* 2003]. To investigate whether increased cell death in *rhoA* morphants was indeed due to enhanced apoptosis, we analyzed the phenotypic embryos by TUNEL assay. It has been shown that the embryonic programmed cell death in zebrafish initiates from the onset of gastrulation [Yabu *et al.* 2001a; Yabu *et al.* 2001b]. Hence, the TUNEL assay was carried out on the embryos from 60% epiboly stage onwards to 2 dpf. The apparently increased apoptosis in *rhoA* morphants was first observed at the end of gastrulation (29/34, Figure 4.2b) while no obvious apoptosis was detected in control MO-injected embryos at this stage (0/44, Figure 4.2a). This result is consistent with previous study that when embryos were treated with various stresses including heat, shock, UV and γ-ray irradiation at 3hpf, the earliest obvious apoptosis was detected at 1-somit stage [Yabu *et al.* 2001b]. Moreover, the stress-induced apoptosis is correlated with enhanced caspase-3-like enzymatic activity in embryos. The consistent appearance of apoptotic cells beginning after gastrulation in both rhoA morphants and stress-treated embryos suggests that the common apoptotic pathway could be specifically activated after gastrulation. Alternatively, the apoptosis could be induced during gastrulation, but the accumulated effects can be obviously detected by Tunel assay after gastrulation. During somitogenesis, only a few scattered apoptotic cells were distributed throughout the control MO-injected embryos (18/21, Figure 4.2c), consistent with the observation by Cole and Ross [Cole *et al.* 2001]. In contrast, apoptosis in *rhoA* morphants became more prominent during early somitogenesis (29/31, Figure 4.2d). By

mid-segmentation stage, much more TUNEL-positive cells were distributed throughout the whole embryo especially in the head and tail (52/52, Figure 4.2f; 33/35, Figure 4.2g; 28/29, Figure 4.2h). The increased apoptosis in *rhoA* morphants was maintained throughout somitogenesis (47/48, Figure 4.2j; 18/18, Figure 4.2k; 23/26, Figure 4.2l). By 2 dpf, the TUNEL-positive cells were detected in forebrain, midbrain, hindbrain (23/24, Figure 4.2n, p, r and t); and particularly concentrated in those tissues known to be highly proliferative during embryogenesis, such as the posterior tectum (Figure 4.2p) and neural retina (Figure 4.2r) [Wullimann *et al.* 2000]. This could result in much smaller head and eyes than those in control MO-injected embryos. In summary, this enhanced apoptosis was correlated with the cell death observed in *rhoA* morphants, suggesting that the developmental defects in reduced body size and shortened body length caused by RhoA knockdown was probably due to increased apoptosis.

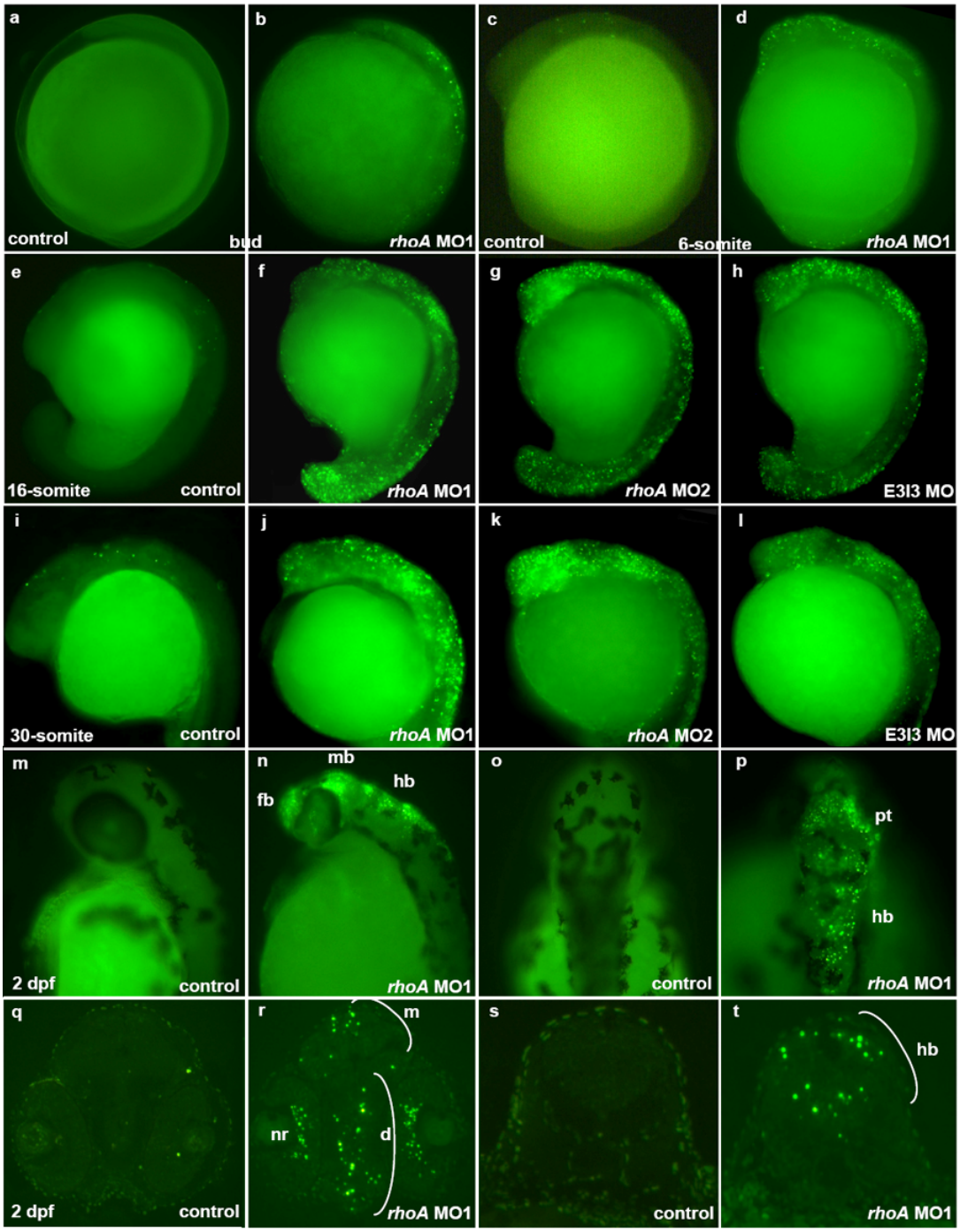

a
control
bud
b
rhoA MO1
c
control
6-somite
d
rhoA MO1
e
16-somite
control
f
rhoA MO1
g
rhoA MO2
h
E3I3 MO
i
30-somite
control
j
rhoA MO1
k
rhoA MO2
l
E3I3 MO
m
2 dpf
control
n
mb
hb
fb
rhoA MO1
o
control
p
pt
hb
rhoA MO1
q
2 dpf
control
r
m
nr
d
rhoA MO1
s
control
t
hb
rhoA MO1

Figure 4.2 RhoA knockdown induces apoptosis during zebrafish embryogenesis. TUNEL assay was performed on embryos injected with control MO (control) and *rhoA* MOs. (a-p) Whole-mount embryos, (a-n) lateral view, dorsal to the right, (o and p) dorsal view, animal pole to the top, (i-l) head region of 30-somite stage embryos, (q-t) anterior cross sections of 2 dpf embryos with dorsal to the top. (e-t) Embryos at the same stage are shown on the same row, and the developmental stages are indicated in the first column. d, diencephalon; dpf, days post-fertilization; fb, forebrain; hb, hindbrain; hpf, hours post-fertilization; m, mesencephalon; mb, midbrain; nr, neural retina; pt, posterior tectum.

To determine the effectiveness of RhoA knockdown by both translation- and splicing-blocking MOs, Western blotting and RT-PCR analysis were performed on the samples extracted from pooled *rhoA* morphants or control MO-injected embryos at 18-somite stage, respectively. As shown in Figure 4.3a, the level of endogenous RhoA was dramatically reduced in either *rhoA* MO1- or *rhoA* MO2- injected embryos, indicating that RhoA translation can be successfully blocked by *rhoA* MO1&2. The E3I3 MO was expected to remove the exon3, resulting in the retention of intron3. This was verified by RT-PCR (Figure 4.3c), and sequencing analysis (data not shown). Thus, E3I3 MO resulted in a frame shift and a premature stop-codon in the transcript, leading to premature termination of RhoA synthesis (Figure 4.3b). To further confirm that the abnormal phenotypes and excessive apoptosis in *rhoA* morphants were due to knockdown of RhoA, we co-injected *rhoA* MO1 with *rhoA* mRNA which had no complementary sequence to *rhoA* MO1 and had been demonstrated previously to successfully rescue gastrulation defects in *rhoA* morphants [Zhu *et al.* 2006b]. Results showed that forced expression of *rhoA* mRNA can rescue both developmental defects and enhanced apoptosis caused by loss of RhoA function (see later in Figure 4.5). In addition, the

pattern of apoptotic cells in *rhoA* morphants partially overlaps with that of *rhoA* expression profile that we reported previously [Zhu *et al.* 2006b]. And the abnormal developmental defects in *rhoA* morphants were distinctly different from those induced by Ras GTPase knockdown (Liu and Low, unpublished data), further supporting that the increased apoptosis in *rhoA* morphants is due to loss of RhoA function rather than unspecific toxicity from the MOs used. Taken together, all these data suggest that knockdown of RhoA induce apoptosis during zebrafish embryogenesis, resulting in reduction of body size and shortening in body length. Since all the *rhoA* anti-sense MOs induced very similar phenotypes and enhanced apoptosis from gastrulation onwards, *rhoA* MO1 was used for the subsequent studies.

4.1.3 RhoA knockdown inhibits Mek/Erk activation

Ras-MAPK pathway is well known for its important role in a multitude of cellular processes, including cell survival, gene expression and cell proliferation [Giehl 2005]. Thus, to investigate whether the regulation of cell survival by RhoA during early embryonic development is through cross talk with Ras-MAPK pathway, Western analysis was performed to examine Mek/Erk activation. As shown in Figure 4.4, the level of phospho-Erk, an indicator of Mek/Erk activation, was significantly reduced in *rhoA* morphants, while the total Erk level remained unchanged. Consistently, the phosphorylation of Mek was also decreased in *rhoA* morphants (data not shown). As a control, neither phospho-p38 nor total p38 showed detectable changes in *rhoA* morphants. Thus, these results suggest that RhoA could be important for the activation of Mek-Erk pathway during zebrafish embryogenesis.

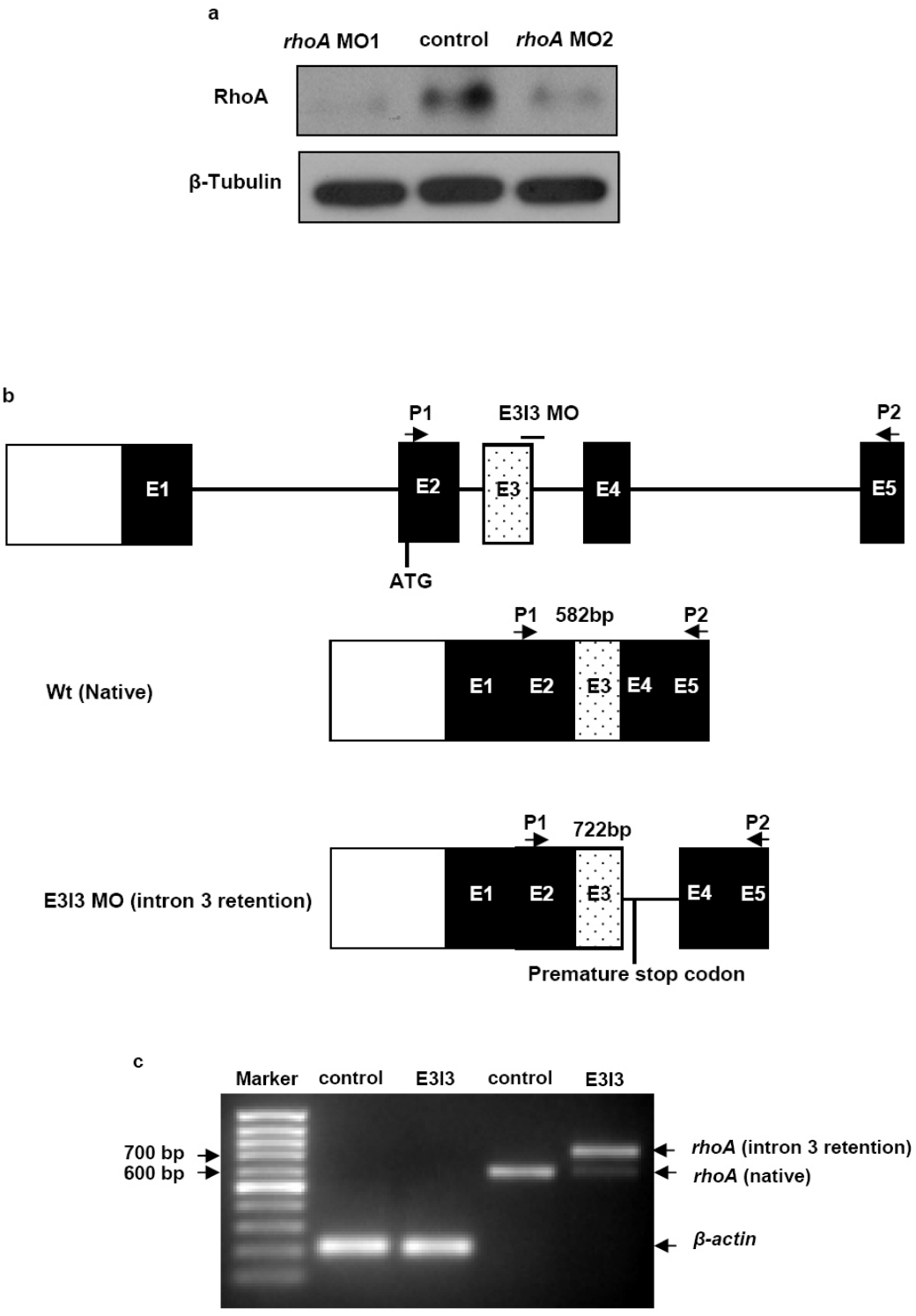

a
rhoA MO1
control
rhoA MO2
RhoA
β-Tubulin
b
P1
E3I3 MO
P2
E1
E2
E3
E4
E5
ATG
P1
582bp
P2
Wt (Native)
E1
E2
E3
E4
E5
P1
722bp
P2
E3I3 MO (intron 3 retention)
E1
E2
E3
E4
E5
Premature stop codon
c
Marker
control
E3I3
control
E3I3
700 bp
600 bp
rhoA (intron 3 retention)
rhoA (native)
β-actin

Figure 4.3 RhoA MOs can elicit RhoA specific knockdown. Embryos injected with control MO (control) and *rhoA* MOs were lysed at 18-somite stage for Western blotting or Semi-quantitative RT-PCR analysis. (a) The protein of endogenous RhoA was dramatically reduced in both *rhoA* MO1&2 injected embryos. (b) Schematic transcript structure of zebrafish *rhoA* gene. P1 and P2 represent the primers for *rhoA* amplification in RT-PCR; the line "-" above E3 highlights the binding site of E3I3 MO. Injection of E3I3 resulted in the retention of intron3, which caused ORF shift and generated a premature stop codon in *rhoA* morphants transcript. (c) RT-PCR analysis of the embryos injected with E3I3 MO (E3I3) or control MO (control). β-Tubulin (a) and *β-actin* (c) show equal loading.

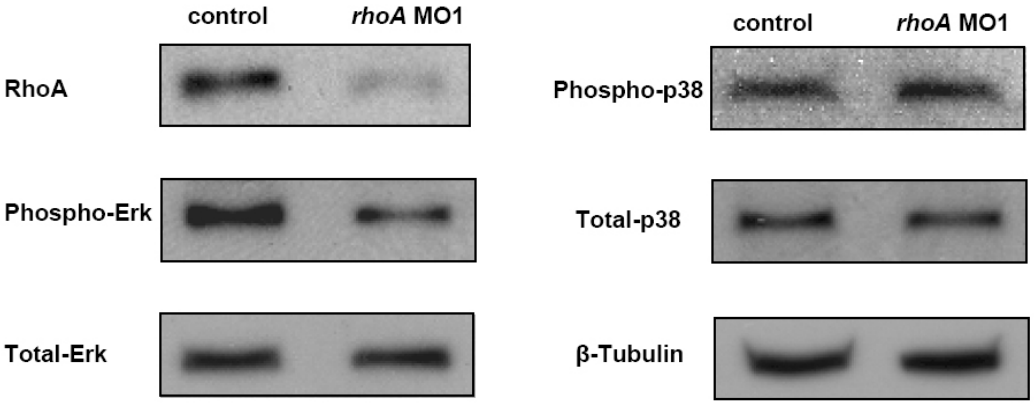
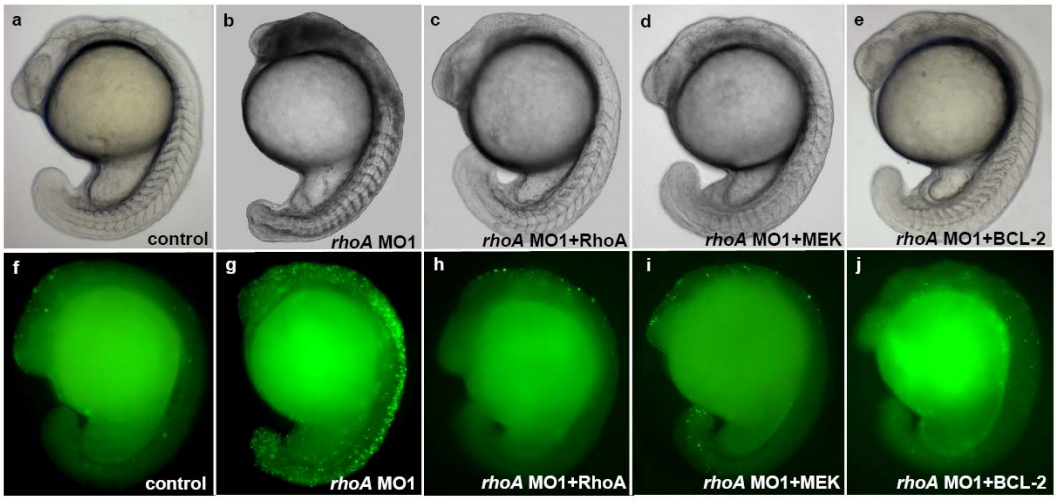
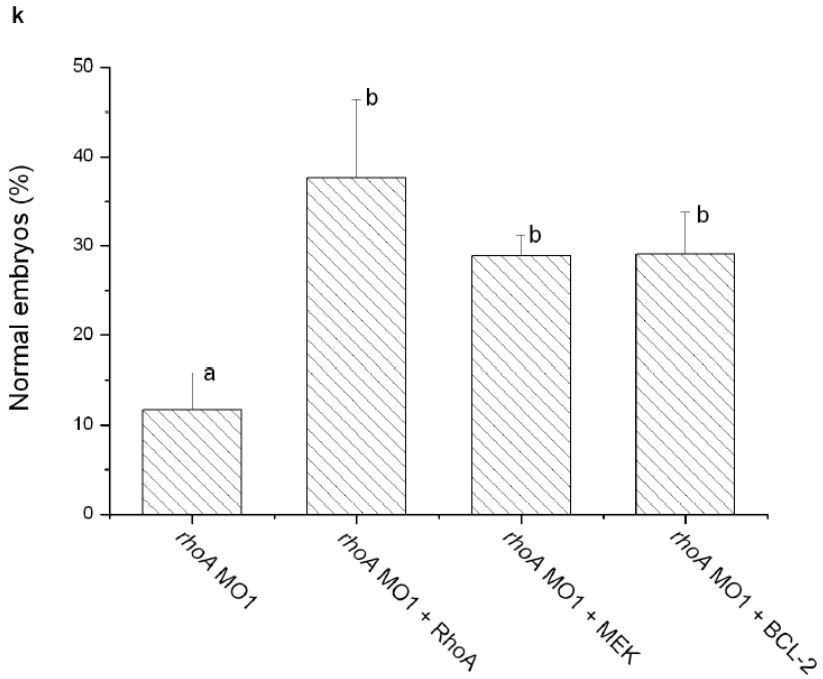


Figure 4.4 RhoA knockdown reduces phosphorylation of Erk. Embryos injected with control MO (control) and *rhoA* MO1 were lysed at 18-somite stage for Western blotting analysis using antibodies against RhoA, MAP kinases, or phospho-MAPKs, as described in "Materials and Methods". β-Tubulin staining shows equal loading.

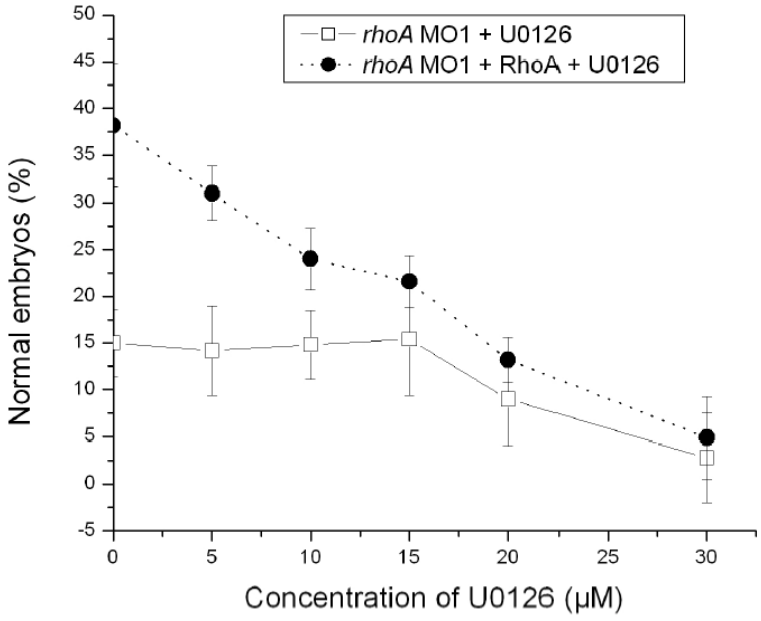
To determine whether the inactivation of Mek-Erk pathway is responsible for the developmental defects and enhanced apoptosis in *rhoA* morphants, we performed a series

of rescue experiments. *In vitro* synthesized mRNA encoding full length zebrafish *rhoA* or mouse *Mek* was titrated and co-injected with *rhoA* MO1 as described in the "Materials and Methods". In *rhoA* MO1-injected group, 88.3% of embryos (n=149) showed "opaque" phenotype with reduced body size and shortened body length during somitogenesis (around 15-18 somites stage) (Figure 4.5b, g and k). In contrast, such developmental defects were corrected in 37.6% of embryos (Figure 4.5c and k, n=156, at $p<0.05$) and the induced apoptosis was dramatically decreased in those phenotypically rescued embryos (42/44, Figure 4.5h) after the forced expression of *rhoA* mRNA. Although the difference between RhoA knockdown and *rhoA* mRNA rescue was statistically significant at $p<0.05$, the percentage of rescue was relatively low. This could be due to the sensitivity of embryos to the co-injection of *rhoA* mRNA with *rhoA* MO1, or the early onset of translation from the rescue mRNA during the early zygote that could derail the proper developmental process. Also, to avoid such possible toxicity and over-expression phenotype caused by high expression of *rhoA*, sub-optimal doses of mRNA was then applied and that might not have been sufficient to fully restore normal development to all the *rhoA* morphants. Alternatively, RhoA was ubiquitously expressed during early zebrafish embryogenesis [Zhu *et al.* 2006b] where the mosaic nature of the mRNA and MO injections as reported by others [McClintock *et al.* 2002; McWhorter *et al.* 2003] may also affect the efficiency of rescue. Similar to *rhoA* mRNA rescue, ectopic expression of *Mek* mRNA not only restored normal development in 28.9% of the embryos (Figure 4.5d and k, n=161, at $p<0.05$), but also prevented apoptosis in 93.6% phenotypically corrected embryos (n=47, Figure 4.5i).

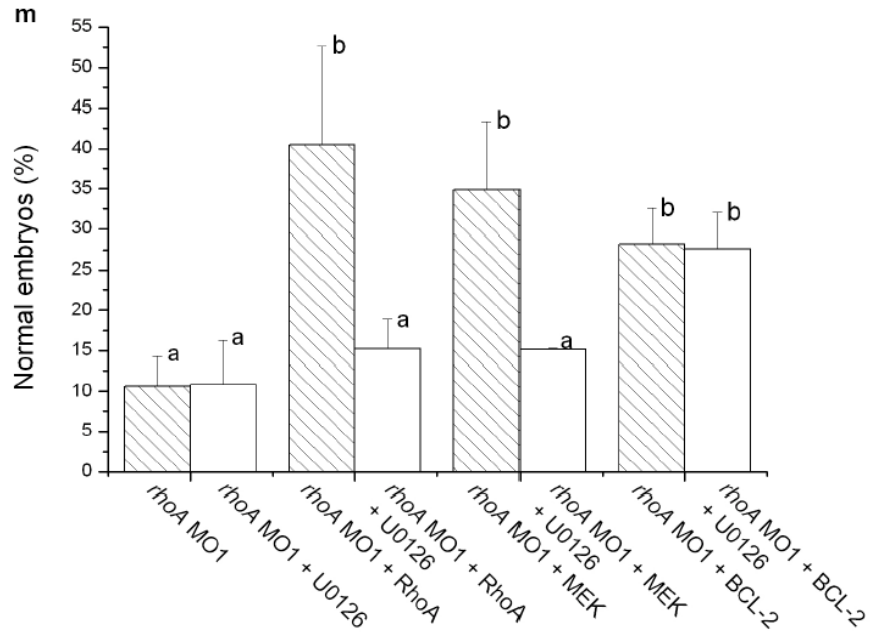

a
control
b
rhoA MO1
c
rhoA MO1+RhoA
d
rhoA MO1+MEK
e
rhoA MO1+BCL-2
f
control
g
rhoA MO1
h
rhoA MO1+RhoA
i
rhoA MO1+MEK
j
rhoA MO1+BCL-2


k
Normal embryos (%)
0
10
20
30
40
50
a
b
b
b
rhoA MO1
rhoA MO1 + RhoA
rhoA MO1 + MEK
rhoA MO1 + BCL-2

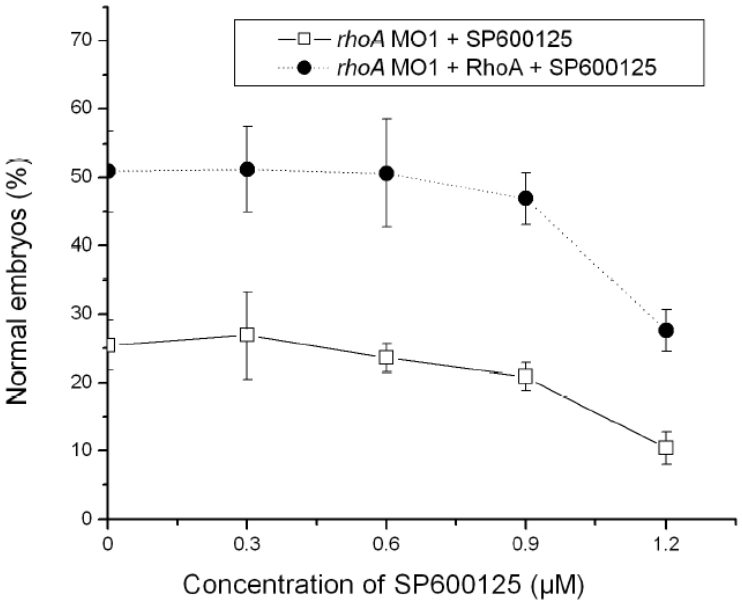
l


rhoA MO1 + U0126
rhoA MO1 + RhoA + U0126
Normal embryos (%)
Concentration of U0126 (μM)
50
45
40
35
30
25
20
15
10
5
0
-5
0
5
10
15
20
25
30

m


Normal embryos (%)
55
50
45
40
35
30
25
20
15
10
5
0
a
a
b
a
b
a
b
b
rhoA MO1
rhoA MO1 + U0126
rhoA MO1 + RhoA
rhoA MO1 + RhoA + U0126
rhoA MO1 + MEK
rhoA MO1 + MEK + U0126
rhoA MO1 + BCL-2
rhoA MO1 + BCL-2 + U0126

n


rhoA MO1 + SP600125
rhoA MO1 + RhoA + SP600125
Normal embryos (%)
Concentration of SP600125 (μM)
0
10
20
30
40
50
60
70
0
0.3
0.6
0.9
1.2

o

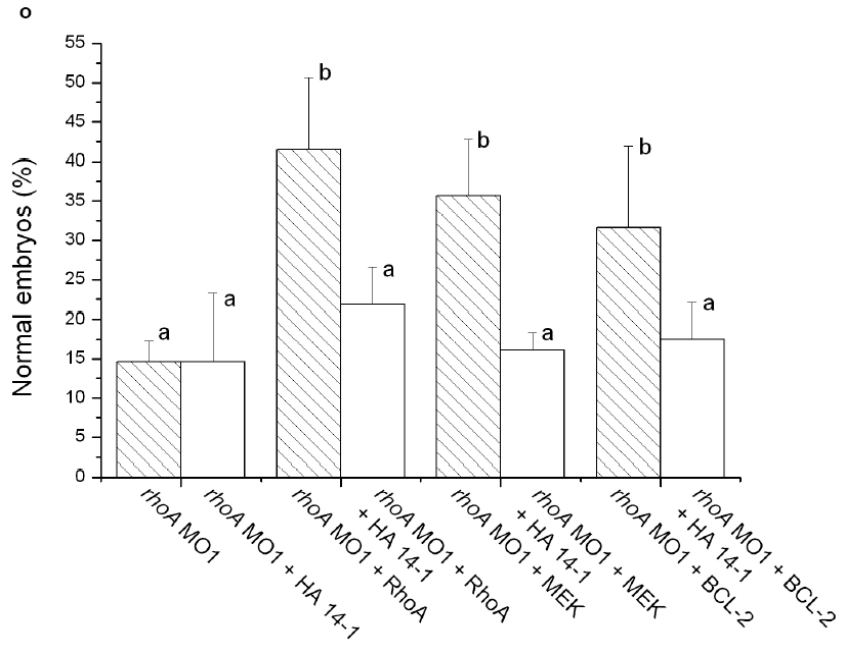
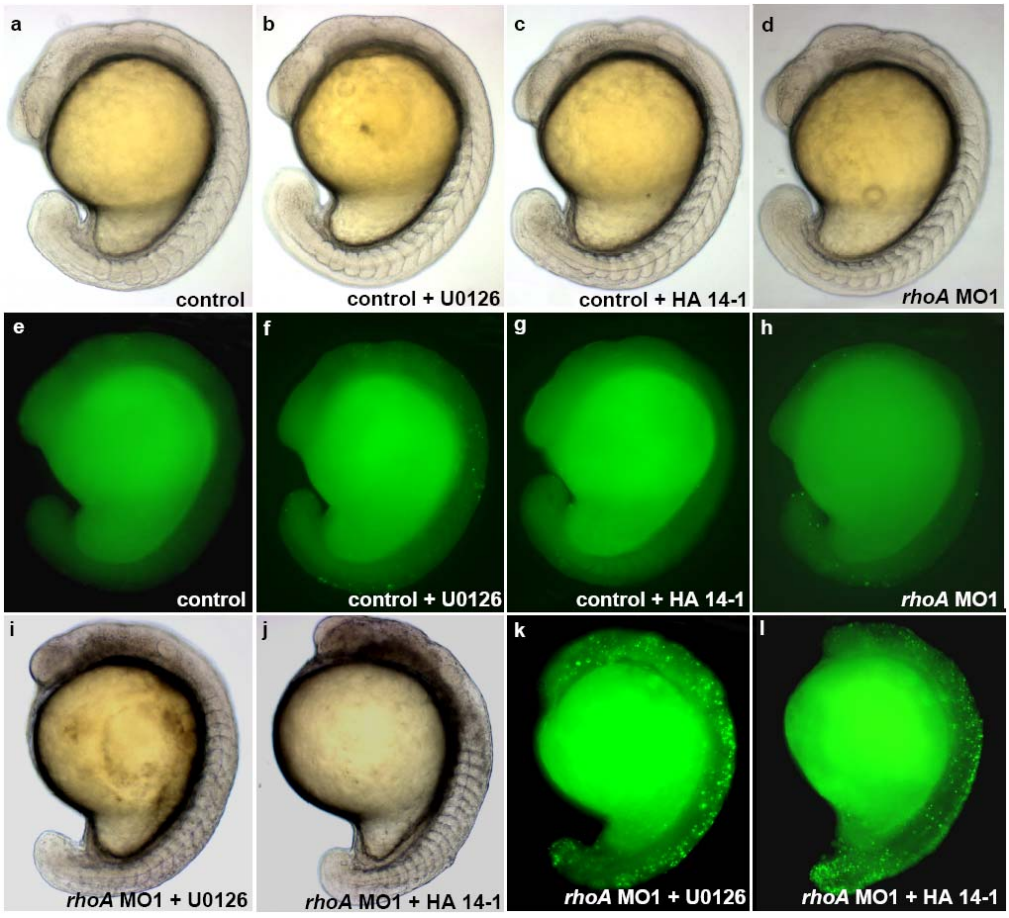

Normal embryos (%)
0
5
10
15
20
25
30
35
40
45
50
55
a
a
b
a
b
a
b
a
rhoA MO1
rhoA MO1 + HA 14-1
rhoA MO1 + RhoA
rhoA MO1 + HA 14-1 + RhoA
rhoA MO1 + MEK
rhoA MO1 + HA 14-1 + MEK
rhoA MO1 + BCL-2
rhoA MO1 + HA 14-1 + BCL-2

Figure 4.5 Mek/Erk and Bcl-2 mediate RhoA signaling for cell survival control. Developmental defects and enhanced apoptosis caused by RhoA knockdown can be rescued successfully by forced expression of mRNA encoding zebrafish *rhoA*, mouse *Mek* and human *BCL-2*. Control MO-injected embryos are indicated as control. (a-e) 18-somite stage embryos, bright field, (f-j) 16-somite stage embryos, TUNEL-positive cells are shown in green dots. (k-o) Percentages of normal embryos injected with *rhoA* MO1 alone or co-injected with *rhoA* MO1 and mRNAs encoding zebrafish *rhoA*, mouse *Mek* or human *BCL-2* in the presence or absence of U0126 at 5-30 μM (l) and 15 μM (m), SP600125 at 0.3-1.2 μM (n), or HA 14-1 at 1.5 μM (o). Different treatments are indicated in the x-axis, each repeated at least three times as described in the "Materials and Methods". Abnormal embryos were scored according to their defects of "opaque" phenotype and overall reduction in body size and body length during somitogenesis (around 15-18 somites stage). Two populations (paired) *t*-test was applied. The difference between a and b is significant at $p<0.05$. Data sharing same letters are not significantly different at $p<0.05$.

To further confirm that Mek/Erk acts downstream of RhoA to promote cell survival, the Mek inhibitor (U0126) [Hong *et al.* 2006] was used to block the activation of Mek/Erk in the embryos which was injected with *rhoA* MO1 alone, or co-injected with *rhoA* MO1 and mRNA encoding either *Mek* or *rhoA*. Serially diluted U0126 was tested on wild type embryos before it was applied on morphants at non-toxic concentration. As shown in Figure 4.5l, *rhoA* mRNA rescue could be inhibited by U0126 in a dose-dependent manner. Compared to the group injected with *rhoA* MO1 alone (10.6% normal, n=251), U0126 abrogated both *rhoA* mRNA (from 40.5% normal, n=219, to 15.2% normal, n=240) and *Mek* mRNA rescue (Figure 4.5m, from 34.9% normal, n=250, to 15.1% normal, n=248, at $p<0.05$). In contrast, no inhibition of *rhoA* mRNA rescue was seen for embryos treated with JNK inhibitor (SP600125) (Figure 4.5n). To further

substantiate the genetic link between RhoA and Mek, low concentration of U0126 and *rhoA* MO1 that would only cause mild developmental defects in few injected embryos were applied together to examine their possible synergy. Compared to the embryos injected with control MO alone (Figure 4.6a, e and m; 0.8% defected, n=67), *rhoA* MO1 alone (Figure 4.6d, h and m; 15.3% defected, n=79) or control MO together with U0126 treatment (Figure 4.6b, f and m; 5.7% defected, n=54), the effects observed upon *rhoA* MO1 injection together with U0126 treatment greatly increased to 52.8% embryos (Figure 4.6i, k and m, n= 108, at $p<0.05$). Taken together, these results demonstrate that RhoA could prevent apoptosis during zebrafish embryogenesis at least through the Mek/Erk pathway.



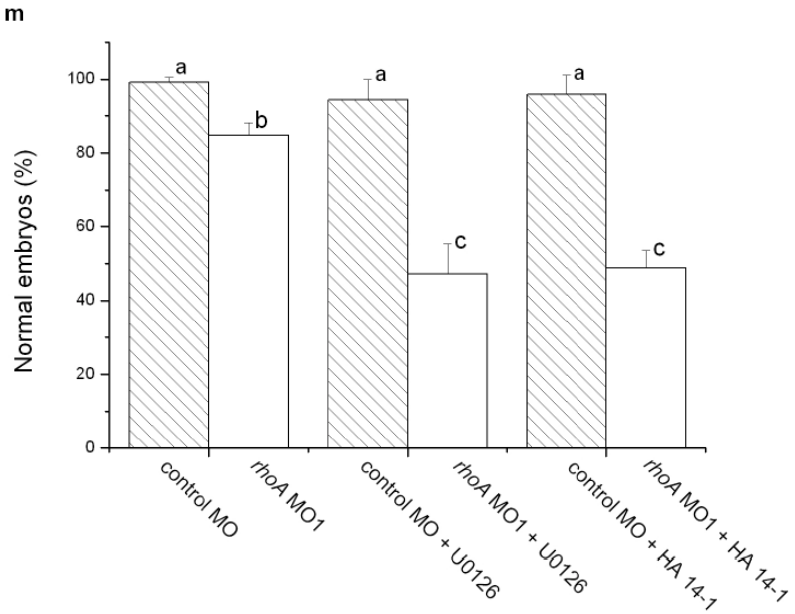


Figure 4.6 Mek/Erk and Bcl-2 act downstream of RhoA to control cell survival. With the combination of low concentration of *rhoA* MO1 or control MO (control) (1.15 ng/embryo) injection and inhibitor treatment (U0126 at 17 μM or HA 14-1 at 1.7 μM), embryos appeared similar developmental defects and increased apoptosis as *rhoA* effective knockdown (at 2.4 ng/embryo). (a-d and i-j) 18-somite stage embryos, bright field, (e-h and k-l) 18-somite stage embryos, TUNEL-positive cells are shown in green dots. (m) Percentages of normal embryos injected with control MO alone, *rhoA* MO1 alone, or in the presence or absence of U0126 or HA 14-1. Different treatments are indicated in the x-axis, each repeated at least three times, where abnormal embryos were scored according to the criteria as mentioned in Figure 4. Two populations (paired) *t*-test was applied. The difference between a, b and c is significant at $p<0.05$. Data sharing same letters are not significantly different at $p<0.05$.

4.1.4 RhoA knockdown suppresses *bcl-2* expression

In addition to activation of Mek/Erk pathway, we wonder whether RhoA may also prevent cells against apoptosis through antagonizing the intrinsic apoptotic pathway. Thus the protein level of Bcl-2, a critical anti-apoptotic gatekeeper for the intrinsic mitochondria pathway, was examined in *rhoA* morphants. Compared to control MO-injected embryos, the protein level of Bcl-2 was significantly reduced in *rhoA* morphants (Figure 4.7). Consistently, ectopic expression of mRNA encoding human *BCL-2* could correct both developmental defects in 29.1% embryos (Figure 4.4e and k, n=186, at $p<0.05$) and increased apoptosis in 97.7% phenotypically rescued embryos (n=43, Figure 4.5j). Besides, antagonizing the anti-apoptotic function of Bcl-2 by its pharmacological inhibitor, HA 14-1 [Wang *et al.* 2000] (at a non-toxic concentration), effectively abolished all functional rescues elicited by *rhoA* (from 41.5% normal, n=149, to 21.9% normal, n=133, at $p<0.05$), *Mek* (from 35.7% normal, n=135, to 16.1% normal, n=106, at $p<0.05$), and *BCL-2* mRNA (Figure 4.5o, from 31.6% normal, n=214, to 17.5% normal, n=168, at $p<0.05$). In contrast, Mek inhibitor, U0126, had no effect on the *BCL-2* mRNA rescue (Figure 4.5m, from 28.2% normal, n=144, to 27.6% normal, n=95). Moreover, combined treatment of HA 14-1 with injection of *rhoA* MO1 at low concentration resulted in “opaque” phenotype, overall reduction of body size and body length, and enhanced apoptosis in 51.1% embryos (Figure 4.6c, g, j, l and m, n=141, $p<0.05$). Supporting this, high dose of HA 14-1 could induce very similar developmental defects as RhoA knockdown (Figure 4.8c). Taken together, these results suggest that Bcl-2, albeit mechanism unknown, may act as downstream response to anti-apoptotic signal from RhoA-Mek/Erk during zebrafish embryogenesis.

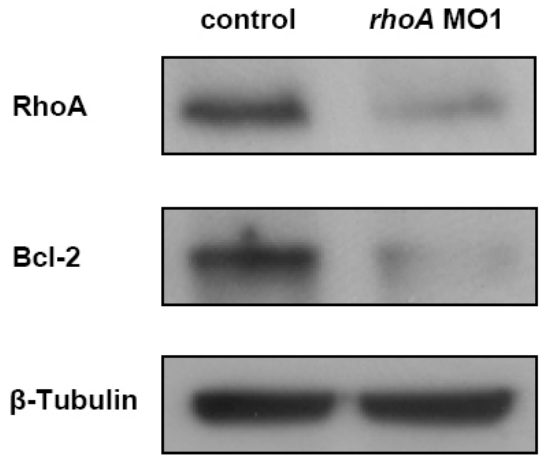


Figure 4.7 RhoA knockdown reduces *bcl-2* expression. Embryos injected with control MO (control) and *rhoA* MO1 were lysed at 18-somite stage for Western blotting analysis using antibodies against RhoA and Bcl-2, as described in "Materials and Methods". β-Tubulin staining shows equal loading.

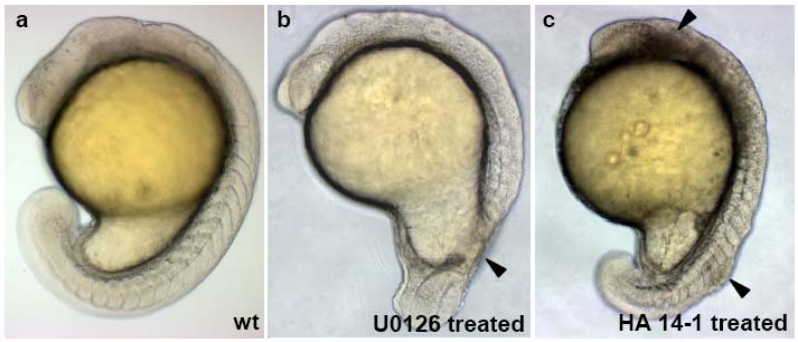


Figure 4.8 Developmental defects caused by strong inhibition of Mek/Erk or Bcl-2 signaling. (a-c) 18-somite stage embryos, lateral view, dorsal to the right, (a) wt, (b) embryos treated with U0126 at 35 μM, (c) embryos treated with HA 14-1 at 2.0 μM. For HA 14-1 treatment, embryos showed similar phenotype as *rhoA* morphants, including opaque region in head, deformed somites, and overall reduction of body size and length. For U0126 treatment, majority of embryos were dead. Few survived embryos showed severe apoptosis mainly at tail region, which led to loss of tail during further development. Scattered apoptosis was also seen in the head region in some embryos (data not shown). Arrowhead indicates the opaque and apoptosis regions.

4.2 Discussion

4.2.1 RhoA controls cell survival via Mek/Erk activation during embryogenesis

Rho GTPases are key regulators for cytoskeletal dynamics, but their involvement in executing apoptosis control during animal development is less well understood. Our present study shows that knockdown of RhoA in zebrafish causes increased apoptosis during embryogenesis, resulting in reduced body size and body length. Interestingly, the zebrafish *rhoA* morphants show some similar developmental defects as *Erk2* knockout mice [Yao *et al.* 2003], but not to knockouts of other *MAPKs* including the *Jnk* and *p38* [Aouadi *et al.* 2006]. Consistently, the activation of Mek/Erk is greatly reduced in *rhoA* morphants, while p38 and Jnk signalings remain unaffected. Moreover, ectopic expression of *Mek* mRNA can faithfully correct such developmental abnormalities in *rhoA* morphants while inhibiting Mek/Erk by U0126 can block *rhoA* mRNA rescue in a dose-dependent manner. Furthermore, combined low dose of *rhoA* MO injection and U0126 treatment can recapitulate the level of apoptosis induced by RhoA knockdown. These data indicate that Mek/Erk activation is required for RhoA signaling to promote cell survival during embryonic development. However, distinct from *Erk2* knockout mice, *rhoA* morphants exhibit unaltered cell fate determination [Zhu *et al.* 2006b], which is consistent with those observation in *Xenopus* [Tahinci *et al.* 2003]. Nonetheless, high dose of U0126 can induce embryonic lethality in the majority of treated embryos and severe apoptosis in few survivors (Figure 4.6b). Thise suggests that strong inhibition on Mek/Erk signaling could cause more severe embryonic defects than RhoA knockdown, reflecting broader biological roles of Mek/Erk in cell proliferation, cell cycle progression and gene transcription [Giehl 2005]. In addition, the genetic link between RhoA and

Mek/Erk pathway can be further supported by their reminiscent expression domain during embryonic development, e.g. *rhoA* [Zhu *et al.* 2006b] and *erk* [Krens *et al.* 2006] are both highly expressed in forebrain, midbrain-hindbrain boundary, hindbrain, eyes and tail region during zebrafish embryonic development. Indeed, the similar expression profile of *RhoA* and the *phosphor-Erk* are very conserved from fly to vertebrate, including *Drosophila* [Gabay *et al.* 1997a; Gabay *et al.* 1997b], *Xenopus* [LaBonne *et al.* 1997; Christen *et al.* 1999; Wunnenberg-Stapleton *et al.* 1999] and mouse [Corson *et al.* 2003], which suggests that the functions of these prauroteins could be linked during embryogenesis.

4.2.2 RhoA prevents Bcl-2-dependent apoptosis via activation of Mek/Erk pathway

Bcl-2, as one of earliest and best characterized anti-apoptotic proteins, plays a central role to inactivate the intrinsic apoptotic pathway by sequestering pro-apoptotic Bcl-2 family proteins (such as Bax) and blocking their targeting to mitochondria for cytochrome c release. Thus the ratio of anti-apoptotic Bcl-2 proteins with pro-apoptotic Bcl-2 family members is crucial for determination of cells surviving [Walensky 2006; Skommer *et al.* 2007]. Increasing evidence has shown that RhoA and Mek/Erk signalings are essential for cell survival by up-regulating the expression of Bcl-2 in a variety of cell lines [Navarro *et al.* 1999; Rios-Munoz *et al.* 2005], and ERK1/2 has been implicated to phosphorylate Bcl-2, leading to its full potency in anti-apoptotic function by stabilizing the Bcl-2-Bax heterodimerization in murine IL-3- dependent myeloid cell lines [Deng *et al.* 2000; Deng *et al.* 2001]. Consistently, our study demonstrates that the inactivation of Mek/Erk pathway and reduced Bcl-2 protein level caused by RhoA knockdown are closely associated with increased apoptosis in zebrafish *rhoA* morphants. Despite this,

our current study cannot rule out the involvement of other anti-apoptotic or pro-apoptotic Bcl-2 family members in RhoA-induced cell survival during zebrafish embryogenesis. This is because the pharmacological inhibitor, HA 14-1, may also inhibit Bcl-2 close homologue, such as Bcl-xL [Castelli *et al.* 2004; Doshi *et al.* 2006]. Besides, RhoA and Mek/Erk have also been implicated to control the expression of other anti-apoptotic Bcl-2 family members, such as Bcl-xL, myeloid cell leukemia-1 (Mcl-1) and pro-apoptotic Bcl-2 family proteins including Bim, Bad and Bax, in a range of cell lines [Craxton *et al.* 2005; Rios-Munoz *et al.* 2005; Betito *et al.* 2006; Del Re *et al.* 2007]. Nonetheless, our study supports the notion that RhoA-Mek/Erk signaling prevents intrinsic apoptosis *in vivo* at least through up-regulating the expression of *bcl-2* (Figure 4.10).

4.2.3 Actin dynamics control by RhoA as a possible link to apoptosis

In addition to regulating the ratio of anti-apoptotic and pro-apoptotic Bcl-2 family proteins, the alteration of actin dynamics induced by RhoA knockdown may also contribute to apoptosis. It has been reported that an intact cytoskeleton mediated by Rho/ROCK is necessary for overall ERK1/2 activation and their nuclear translocation, as well as activation of transcription in SDF-1-stimulated cells [Zhao *et al.* 2006], whereas disruption of the actin cytoskeleton by inhibition of ROCK results in the induction of apoptosis in airway epithelial cells [Moore *et al.* 2004], human endothelial cells [Li *et al.* 2002] and cytotoxic T lymphocytes [Subauste *et al.* 2000]. Similarly, disruption of actin cytoskeleton can also induce apoptosis mediated by Bcl-2 in MEK-transformed EpH4 and MCF10A mammary epithelial cells [Martin *et al.* 2001; Pinkas *et al.* 2004]. On the contrary, stabilization of actin cytoskeleton by down-regulation of the actin severing protein, gelsolin [Harms *et al.* 2004] or by addition of jasplakinolide that induces the

accumulation of large F-actin aggregates can also lead to increased apoptosis in various cells [Posey *et al.* 1999; Odaka *et al.* 2000]. These data suggest that alterations in actin dynamics, either disruption or stabilization of actin cytoskeleton, could result in apoptosis. As such, this may help to explain the paradox that both down-regulation and over-expression of RhoA could induce apoptosis in different cellular contexts.

4.2.4 Cell survival is uncoupled from gastrulation control by RhoA

Besides the prevention of cell death, cytoskeleton rearrangement regulated by RhoA during cell movements may also be mediated by Mek/Erk and Bcl-2 signaling pathways. It has been shown that Mek can enhance myosin light chain kinase (MLCK) activity and lead to phosphorylation of myosin light chains (MLC) in the control of cell's motility [Klemke *et al.* 1997], while over-expression of Bcl-2 can inhibit actin depolymerisation to promote cell migration in myelocytic cell lines [Korichneva *et al.* 1999]. Moreover, over-expression of Mek or Bcl-2 has also been reported to enhance the migration of a variety of cells including endothelial cells [Rikitake *et al.* 2000], human breast cancer cells [Del Bufalo *et al.* 1997], human bladder cancer cells [Miyake *et al.* 1999], and glioma cells [Wick *et al.* 1998]. Consistently, our study shows that forced expression of either Mek or BCL-2 can correct gastrulation defect in *rhoA* morphants (Figure 4.9), suggesting Mek and Bcl-2 could act downstream of RhoA to control cell movement during zebrafish gastrulation. However, results from our splicing MO experiments revealed that knockdown of zygotic RhoA exhibit only reduced body size/length with increased apoptosis without any gastrulation movement defects. Therefore, our results imply that although these three proteins are important in both

processes, the mechanisms of their actions during CE movement and cell survival are likely to be different and could be uncoupled (Figure 4.10).

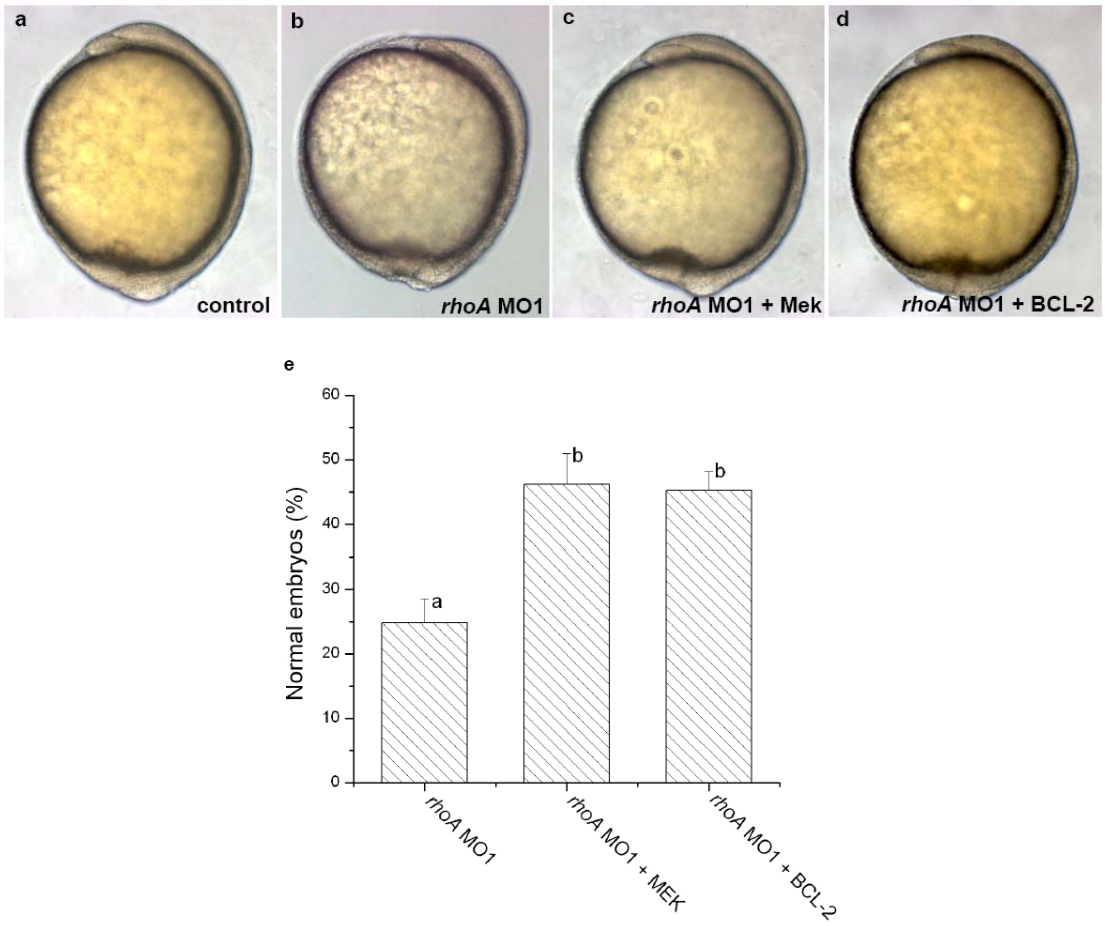
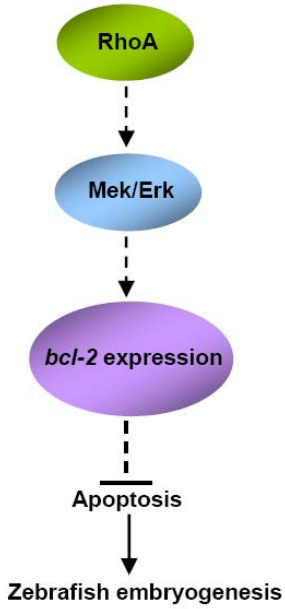


Figure 4.9 Mek/Erk and Bcl-2 mediate RhoA signaling for gastrulation cell movement. Gastrulation movement defect caused by RhoA knockdown can be rescued successfully by forced expression of mRNA encoding mouse *Mek* and human BCL-2. Control MO-injected embryos are indicated as control. (a-d) Tail bud stage embryos, lateral view, dorsal to the right, (e) Percentages of normal embryos injected with *rhoA* MO1 alone or co-injected with *rhoA* MO1 and mRNAs encoding mouse *Mek* or human BCL-2. Different treatments are indicated in the x-axis, each repeated at least three times as described in the "Materials and Methods", where abnormal embryos were scored according to the defects in shortened anterior-posterior body axis. Two populations (paired) *t*-test was applied. The difference between a and b is significant at $p<0.01$. Data sharing same letters are not significantly different at $p<0.05$.

Figure 4.10 RhoA prevents apoptosis by activation of Mek/Erk pathways.



During zebrafish embryogenesis, the anti-apoptotic effect of RhoA is elicited through activation of Mek/Erk signaling, which could antagonize the mitochondria-mediated intrinsic apoptotic pathway at least *via* the up-regulation of *bcl-2* expression. Dotted lines indicate the effects between components, but the nature of their interaction (e.g. indirect or direct) remains to be investigated.

4.3 Conclusion

In summary, we have uncovered the genetic link between RhoA and Mek/Erk signaling as well as Bcl-2 *in vivo*, where RhoA prevents Bcl-2-dependent intrinsic apoptosis *via* activation of Mek/Erk pathway. This could pave the way to our better understanding of regulation for apoptosis by these key proteins and their related members during normal development and pathophysiological conditions.

Chapter 5 Concluding remarks

5.1 Conclusions and contributions

By using a combination of gene suppression and functional rescue experiments together with specific pharmacological inhibitors, we have demonstrated that RhoA is important for both cell movement and cell survival during zebrafish embryogenesis via different downstream signaling cascades. During zebrafish gastrulation, the basic body plan is established through four types of cell movements, namely epiboly, internalization, convergence and extension (CE). In our study, we find that RhoA acts downstream of non-canonical Wnt5 and Wnt11 to regulate CE movements, without affecting cell fate determination. This result not only extends our knowledge of the molecular mechanism underlying the gastrulation movements, it also supports the notion that the overlapping function of Wnt5 and Wnt11 in trunk region could be due to common downstream targets during zebrafish gastrulation. Besides, our study shows that the effect of RhoA on convergence and extension movements is elicited by two of its key downstream effectors, Rock and Dia, cooperatively. This is for the first time to demonstrate the cooperation between Rock and Dia in the control of cell movements *in vivo*, suggesting that zebrafish could be an excellent and powerful model system for further investigation of the roles of various classes of small GTPases in regulating cell dynamics *in vivo*. In addition to the control of CE movement, RhoA also plays a critical role in the prevention of cells from apoptosis during embryogenesis through activation of Mek/Erk signaling pathway and upregulation of *bcl-2* expression. Moreover, ectopic expression of mRNA encoding for either *MEK* or BCL-2 can rescue gastrulation movement's defects in *rhoA* morphants

sucessfully, suggesting that the Mek/Erk and Bcl-2 signaling are also involved in CE movement control during zebrafish gastrulation. However, knockdown of zygotic RhoA exhibits only reduced body size/length with increased apoptosis without any gastrulation movement defects, implicating that the cell survival is uncoupled from gastrulation control by RhoA. Taken together, our study has not only demonstrated the importance of RhoA in cell movements and cell survival *in vivo*, but also established a good *in vivo* model for better understanding of the regulation of these two processes by these key proteins (RhoA, Mek and Bcl-2) and their related members during normal development and pathophysiological conditions.

5.2 Limitations

The limitation of our study is mainly the result of inherent problems with the experimental model system and the morpholino (MO) technique employed. Zebrafish has been extensively accepted as an excellent model system for developmental and genetic studies, and MO is well known to mediate gene specific functional knockdown by either blocking gene translation or disrupting mRNA splicing. However, both of them have drawbacks. For example, similar to other experimental systems, maternally inherited gene products in zebrafish can often mask the effects elicited by MO. Our study and other's work have shown that zebrafish RhoA protein is maternally provided. Although two non-overlapping translation-blocking MOs and one splicing-blocking MO can successfully disrupt *rhoA* translation or splicing, they have no effect on the maternal RhoA protein. Moreover, it is difficult to determinate how long the maternal RhoA protein lasts during embryonic development, because of the early and constant

expression of zygotic *rhoA*. Hence, the role of RhoA during very early embryogenesis could not be addressed by MO-based functional knockdown.

Other MO drawbacks include short lifetime, no tissue or cell type specific inhibition, mis-targeting and toxicity. In general, MO can last for around 3-5 days, and it is normally microinjected into embryos at one-cell stage for even distribution. However, the ubiquitous inhibition of RhoA function in early embryogenesis by MO leads to lethality of majority of the morphants within 4 dpf. Therefore, the role of RhoA in later stage embryonic development could not be addressed in the current study. Recently, the mis-targeting and toxicity of MOs has attracted more and more attention. To avoid these possibilities, three RhoA-specific antisense MOs, one 5 bp-mismatch MO and one standard control MO combined with mRNA rescue have been applied. Although substantial evidence suggests that the developmental defects in *rhoA* morphants is due to knockdown of RhoA function, the effect of mis-targeting and toxicity of MOs could still not be completely ruled out.

5.3 Suggestions for future studies

In our study, we have demonstrated that RhoA is essential for both gastrulation movements and cell survival during zebrafish embryogenesis, which could be elicited by distinct downstream signaling pathways. Combined with previous studies in different model systems, all these experimental evidence reveals that RhoA plays widespread and diverse functions in multiple developmental processes. Therefore, how RhoA coordinately affects such distinct morphogenetic events in different developmental stage and tissue contexts of multi-cellular organisms needs to be further elucidated. One of the

possibilities could be the precise temporal and spatial regulation by its upstream regulators, such as the GAPs and GEFs. In mammals, more than 70 RhoGAPs and 80 RhoGEFs have been identified, that presumably serve to regulate the 23 Rho proteins. This relatively large number of regulatory proteins could tightly control the activation of RhoA in particular regions at predetermined stages to elicit appropriate downstream responses. In addition, temporal-spatial expression of the multiple RhoA effectors may also contribute to diverse developmental functions mediated by RhoA. Therefore, detailed characterization of temporal and spatial expression profiles of each of the GAPs, GEFs and effectors for Rho GTPases during development, as well as their sub-cellular distribution during developmental processes would be great help in dissecting the molecular mechanism of Rho-mediated diverse functions during different developmental processes and exploring cell lineage or tissue specific role of individual Rho proteins.

Two major downstream effectors of RhoA, namely, Rock and Dia, have been shown to control cell migration by cooperative regulation of actin cytoskeleton rearrangement and microtubule stabilization *in vitro*. In our study, we have demonstrated that Rock and Dia coordinately elicit RhoA-mediated Wnt5 and Wnt11 signaling during zebrafish gastrulation movements, but that specific regulation of actin and microtubule dynamics in this process is still unclear. We also show that Mek/Erk and Bcl-2 can act downstream of RhoA to control both gastrulation movement and apoptosis during zebrafish embryogenesis. Although, accumulating studies suggest that actin-cytoskeleton arrangement could be involved in cell movements and apoptosis *in vitro*, the role of actin dynamics in these two processes *in vivo* remain largely unknown. Therefore, given the availability of actin- GFP/RFP and microtubule-GFP/RFP stable transgenic lines, the

optical transparent of zebrafish embryos during early development and the amenability to various cellular, molecular and genetic techniques, zebrafish could be an ideal model to address these questions in future.

Besides the above, our current study focuses mainly on the understanding of the signaling mechanism underlying the role of RhoA in embryonic development. We have revealed the genetic links and functional relationship between RhoA and Wnt5/Wnt11 in the CE movements, and, RhoA with Mek/Erk as well as Bcl-2 in the cell survival during zebrafish embryogenesis. However, how are these signaling pathways linked? Are Rock, Dia and Mek/Erk as well as Bcl-2 cooperatively regulated in these two processes? Could RhoA activate Mek/Erk or Bcl-2 directly by forming a complex or indirect via other components? All these questions need to be further addressed.

In addition to the understanding of the molecular basis of RhoA in cell movement and cell survival control during development, our current findings have immediate implications on the potential role of Rho GTPases in various pathophysiological settings including tumorgenesis that is closely associated with reduced cell death, increased cell proliferation and enhanced cell motility. Indeed, deregulation of RhoA activity has been detected in a variety of cancers in colon, breast, lung, testicular germ cell, head, and liver [Sahai *et al.* 2002a], while other close homologs such as RhoB and RhoC are also implicated to be cancer-promoting [Wheeler *et al.* 2004]. With the concerted regulation of Mek/Erk and Bcl-2 by RhoA as presented here, one would envisage that these tripartite signaling nodes should provide attractive alternatives for therapeutic intervention. Already, in acute myelogenous leukemia, synergistic induction of apoptosis

was achieved by simultaneous disruption of the Bcl-2 and MEK/MAPK pathways [Milella *et al.* 2002], and RhoA GTPase inactivation by lipophilic statins induces osteosarcoma cell apoptosis through a caspase-dependent process by suppressing the ERK and BCL-2 signaling [Fromigue *et al.* 2006]. Besides the above, several stable transgenic lines with the overexpression of Rho GTPases specific in liver have been generated in our laboratory to investigate their function in tumorgenesis such as hepatocarcinoma. We hope that these stable transgenic lines would be valuable *in vivo* models for better our understanding of the cellular and molecular basis underlying tumorgenesis and malignancy, and for high-throughput pharmacological compounds screening in the discovery of the potential and promising anticancer drugs.

Although our study has shed some light on the importance of RhoA during normal embryonic development, many questions remain to be addressed. With increasing findings about Rho small GTPases obtained from different model systems, their roles in physiological and pathological processes will be better understood, and to enable us to gain deeper understanding of the mechanism underlying related disease and to identify more effective therapeutic approaches.

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