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par

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Contrôle des mouvements cellulaires et de la mise en place du patron embryonnaire précoce par les facteurs Angiomotin like 2 et FrzA chez l'embryon de poisson zèbre (*Danio rerio*)

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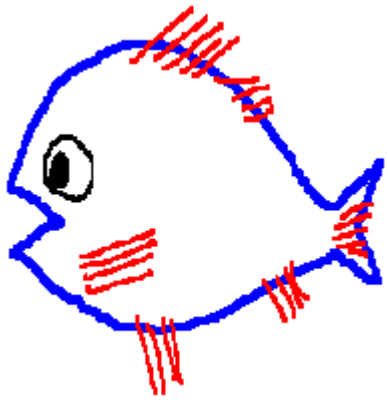


"Rôle des facteurs Angiomotin like2 et FrzA dans le contrôle de la mise en place du patron embryonnaire et des movements cellulaires au cours du développement embryonnaire précoce du poisson zèbre (*Danio rerio*)"

Introduction Générale

A - Le poisson zèbre, un modèle d'étude du développement embryonnaire

Le poisson zèbre Danio rerio a été choisi pour cette étude car il présente de nombreux avantages techniques qui le rendent particulièrement attractif pour l'analyse moléculaire du développement embryonnaire précoce. De petite taille et très prolifique, son développement est externe et l'embryogenèse est très rapide (48h). Bien que son temps de génération ne soit pas particulièrement court (2-4 mois) le poisson zèbre a été utilisé pour une dissection génétique du développement de l'embryon.

B - Les différents movements cellulaires durant le développement précoce du poisson zèbre

L'oeuf subit initialement une série de clivages, qui conduit rapidement à la formation d'une masse de 512 cellules sans que le génome zygotique ne soit activé. A ce stade, connu sous le nom de transition mid-blastuléenne, lors du 10ème cycle cellulaire le génome du zygote commence à être transcrit. Peu après, la masse de cellules présente au futur pole animal de l'embryon commence à subir différents mouvements cellulaires: l'intercalation radiaire, le

movement d'épibolie, puis lorsque ce dernier aura conduit à la couverture de 50% de la réserve vitelline, le début de la gastrulation est caractérisé par un mouvement d'involution des cellules qui vont former l'hypoblaste et un mouvement de convergence-extension qui va conduire à la formation de l'embryon sur la face dorsale ainsi qu'à son élongation suivant l'axe antéro-postérieur.

C - Facteurs impliqués dans les movements cellulaires

De nombreuses études antérieures réalisées tant sur des cellules en culture que sur d'autres modèles animaux ont permis de mettre en évidence un grand nombre de facteurs impliqués dans la migration des cellules. Une analyse génétique réalisée chez le poisson zèbre a mis en évidence des facteurs contrôlant les movements d'épibolie anisi que les movements d'internalisation des cellules. De même les facteurs impliqués dans la voie de signalisation Wnt non canonique ont montré contrôler les movements de convergence-extension alors que ceux de la voie β-catenin-Stat3-Liv1-Snail1 contrôlent la transition epithelio mésenchymateuse qui est requise pour ces movements.

D - Facteurs impliqués dans la détermination cellulaire

En plus de leur capacité migratoire les cellules de l'embryon acquièrent une identité propre au cours des étapes de détermination cellulaire. Cette phase du développement fait intervenir des voie de signalisation impliquant des facteurs TGF beta tels que les BMP (Bone morphogenetic proteins) et Nodal, les facteurs Wnt ainsi que les FGF (Facteurs de croissance fibroblastiques). Ces signaux agissant de manière coordonnée vont conduire à

l'établissement de la polarité dorso-ventrale et de la polarité antéro-postérieure.

E - But de mon travail de thèse

Au cours de mon travail de thèse j'ai étudié la fonction de deux facteurs liés à deux voies de signalisation contrôlant le développement de l'embryon, Angiomotin like 2 qui est controlé par la voie FGF et FrzA qui est lié au contrôle de la voie Wnt dans les processus de migration ainsi que de determination cellulaire.

1ère Partie : Rôle d'Angiomotin like 2 (Amotl2) au cours du développement précoce du poisson zèbre

A - Introduction

- Angiomotin like 2 est un gène cible de la signalisation FGF

Ce projet a été initié via une coopération avec le groupe d'Anming Meng de l'Université Tsinghua de Beijing. Nous avons identifié au cours d'un crible des gènes cibles induits par la signalisation FGF. Parmi ces derniers nous avons isolé le gène Angiomotin like 2. Cette protéine fait partie de la famile d'Angiomotin et comprend un domaine "coiled-coil" vraisemblablement impliqué dans des interactions protéines-protéines, un domaine riche en glutamine ainsi qu'un domaine de liaison au motif PDZ mais pas le domaine de liaison à l'angiostatine qui est spécifique d'Angiomotin.

- Rôle d'Angiomotin in vivo and in vitro

Les données générées au cours de l'étude de la fonction d'Angiomotine chez la souris ont permis de montrer que ce facteur était essentiel pour les mouvements de l'endoderme viscéral ainsi que pour la migration des cellules épithéliales. De plus la surexpression d'angiomotin affecte les jonctions cellules cellules.

Cependant angiomotin et angiomotin like 1 ou angiomotin like 2 diffèrents dans différents domaines protéiques et il n'y a aucune donnée concernant le lien entre la fonction d'angiomotin like 2 et sa relation avec les mouvements cellulaires ou les jonctions cellulaires. C'est pourquoi je me suis attaché à analyser via des expériences in vivo la fonction d'amotl2 tant au niveau de ses effets potentiels sur les mouvements cellulaires que sur les conséquences de

cette fonction sur la mise en place du patron embryonnaire lors des étapes de détermination.

B. Resultats

1. Relations entre Angiomotin like 2 et la migration cellulaire.

a. Article: Amotl2 is essential for cell movements in zebrafish embryo and regulates c-Src translocation

Résumé:

Angiomotin (Amot), le membre fondateur de la famille de Motin, est impliqué dans l'angiogenèse en réglant la motilité endothéliale des cellules, et est exigé pour le mouvement de l'endoderme viscéral chez la souris. Cependant, peu de données sont connues au sujet des fonctions biologiques les deux autres membres de la famille de Motin, Angiomotin-like1 (Amotl1) et Angiomotin-like2 (Amotl2). Nous avons identifié chez le poisson zèbre le gène amotl2 en tant que cible de la signalisation FGF. Le gène amotl2 est exprimé tant maternellement que zygotiquement de manière tissu spécifique. La perte de fonction d'Amotl2 conduit à un retard dans le mouvement d'épibolie et affecte le mouvement de convergence et d'extension et les cellules déficientes pour Amotl2 chez des embryons mosaïques ne migrent pas de manière appropriée. Ceci coïncide avec la perte de protrusions membranaires et une désorganisation de l'actine-F. Amotl2 co-localise partiellement avec les endosomes positifs pour RhoB ou EEA1 ainsi qu'avec la tyrosine kinase c-Src. Nous démontrons de plus qu'Amotl2 interagit préférentiellement avec et facilite

la translocation membranaire de la forme phosphorylé de c-Src qui en retour va réguler l'architecture membranaire. Ces données fournissent la première preuve de la fonction essentielle d'Amotl2 pour les mouvements cellulaires chez les vertébrés.

b. Résultats complementaires

1 - Relation entre Amotl2, la signalisation BMP et la migration cellulaire

Amotl2 est requis pour la migration des cellules dans l'embryon de poisson zèbre. Ce gène est sous le contrôle de la voie de signalisation Fgf qui ont été montré être impliqués dans le contrôle de la transcription des facteurs ventralisants BMP. Or les BMP sont également requis pour les mouvements cellulaires dans l'embryon. En effet des cellules injectées avec un ARN codant pour une forme tronquée du récepteur au BMP perdent leur capacité à migrer dorsalement suggérant qu'amotl2 puisse agir sur la migration cellulaire de manière indirecte via un effet sur la voie de signalisation BMP. Néanmoins, cette hypothèse a pu être infirmée en montrant que la stimulation autonome cellulaire des cellules Amotl2- par une forme active de smad1 est incapable de restaurer la migration des cellules montrant que le défaut de migration des morphants Amotl2 n'est pas du à une inactivation de la voie BMP.

2 - Relation entre Amotl2 et la mise en place du patron embryonnaire

a. Amotl2 est nécessaire à la mise en place du patron embryonnaire

En plus des effets observés de la perte de fonction d'Amotl2 sur les mouvements cellulaires de l'embryon j'ai pu mettre en évidence un effet

d'Amotl2 sur la mise en place du patron embryonnaire caractérisé par une extension des territoires dorsaux et une réduction concomittante des territoires ventraux.

b. La perte de fonction d'Amotl2 affecte le patron embryonnaire avant l'initiation des étapes de migration cellulaire

Les défauts dans la mise en place du patron embryonnaire suite à la perte de fonction d'Amotl2 apparaissent dès le stade blastula, c'est-à-dire avant que les mouvements cellulaires n'aient commencé. Je peux donc en conclure que ces défauts ne sont pas une conséquence indirecte des anomalies de migration cellulaire mais précèdent ces derniers.

c. Analyse de la perte de fonction d'Amotl2 dans des clones cellulaires

J'ai ensuite analysé les effets autonome cellulaire d'Amotl2 dans des clones cellulaires et j'ai ainsi pu montrer l'induction de l'expression du facteur dorsalisant chordin dans des clones à la marge ventrale de l'embryon au stade blastula. Ces defauts pourraient résulter d'une diminution de l'activité zygotique de Wnt8 conduisant à une perte des mecanismes ventraux permettant l'inhibition des gènes dorsaux de l'embryon.

d. Lien entre Amotl2 et la sécrétion de Wnt8

J'ai testé l'hypothèse précédente en étudiant la signalisation de Wnt8 au niveau de la sécrétion de ce facteur en faisant l'hypothèse que Amotl2 pourrait plus vraisemblablement affecter des éléments structuraux de la cellule (tels que ceux affectant l'exocytose, l'endocytose) que contrôler directement les élements aval de la voie de signalisation Wnt. De fait, mes résultats

préliminaires mettent en evidence une anomalie de sécrétion de Wnt8 qui tend à s'accumuler dans le noyau des cellules.

e. Sauvetage partiel des défauts de mise en place du patron embryonnaire par surexpression de BMP

Parallèlement à cette étude de la voie de signalisation Wnt j'ai cherché à savoir si les défauts de mise en place du patron embryonnaire pouvaient être corrigés par la stimulation de la voie BMP et j'ai pu constater un sauvetage, au moins partiel de ces défauts suite à l'injection d'ARN codant pour BMP. Ceci suggère que le défaut majeur de mise en place du patron correspond à l'expression ectopique, notamment chordin en position ventrale des facteurs dorsaux.

2ème Partie : Role de FrzA (SFRP1, Secreted Frizzled Related Protein 1) au cours du développement embryonnaire précoce

A. Introduction

1. Isolement du gène FrzA au cours d'un crible par hybridation in situ

Le gène FrzA a été isolé au cours d'un crible par hybridation in situ dont le but est la caractérisation des gènes dont l'expression est spatialement restreinte au cours du développement embryonnaire.

2. Les molecules de la Famille SFRP

Le gène FrzA appartient à la famille des proteines SFPR, c'est-à-dire de proteines sécrétées présentant des similarités structurales avec les protéines

Frizzled, les récepteurs membranaires des facteurs de la famille Wnt. Ces protéines sont caractérisées par la présence d'un domaine riche en cystéine (cystéine rich domaine: CRD). Leur fonction a été très largement étudiée cependant la fonction moléculaire et la contribution de SFRP1/FrzA durant le développement embryonnaire précoce n'a pas encore été élucidé. C'est pourquoi j'ai analysé la fonction et les effets de FrzA sur la mise en place du patron de l'embryon ainsi que sur le contrôle des mouvements cellulaires chez l'embryon de poisson zèbre.

B. Résultats

1 - Structure et patron d'expression de FrzA chez l'embryon de poisson zèbre

Le gène FrzA de poisson zèbre a été isolé et le cDNA correspondant code pour une protéine de 296 acides aminés présentant 65.7% d'identité avec le gène FrzA. Les domaines spécifques de cette protéine tel que le domaine CRD sont conservés et la construction d'un arbre phylogénique des protéines de la famille SFRP démontre que FrzA de poisson zèbre est bien l'orthologue des autres gènes FrzA précédement décrits. Le patron d'expression de ce gène est complexe et très dynamique. On peut cependant noter qu'aux stades précodes du développement FrzA apparait comme exprimé maternellement. Son expression zygotique débute au stade sphère, immédiatement après le début de l'expression du génome zygotique et elle est restreinte au région non marginale de l'embryon, correspondant donc au futur ectoderme.

2 - Effet de la surexpression et de la perte de fonction de FrzA

L'analyse morphologique de la perte de fonction de FrzA par "morpholino knock-down" revèle des défauts de mouvements cellulaires, en particulier du mouvement d'épibolie et des anomalies dans les mouvements de convergence et extension. Parallèlement, une analyse morphologique des phénotypes de gain de fonction de FrzA par injection d'ARN sens révèle que les embryons sont tronqués dans la partie postérieure, et présentent un phénotype de cyclopie. Ces phénotypes peuvent résulter de modifications affectant les mouvements cellulaires ou de modification de la mise en place du patron embryonnaire.

L'étude de ces phénotypes à des stades plus précoces du développement avec des marqueurs moléculaires correspondant à des gènes cibles des voies de signalisation BMP et Wnt8 a été réalisée. Il ressort de cette étude que les hypothèses précédemment faites pour l'action moléculaire de FrzA ne sont pas en accord avec mes observations et suggèrent que FrzA agisse initialement au niveau de l'activité de la b-caténine maternelle. La perte de fonction de FrzA conduit à une augmentation de l'expression de chordin alors que le gain de fonction conduit à sa diminution.

3 - FrzA et la signalisation BMP

Les phénotypes de perte de fonction de FrzA qui conduisent notamment à une activation ectopique de chordin peuvent être partiellement sauvés via une atténuation de l'activité de ce facteur dorsalisant par la technique de "morpholino knock down". De plus les mêmes sauvetages peuvent être obtenus par surexpression des facteurs ventralisant BMP, via l'injection d'ARN sens codant pour ces protéines. Cependant, au delà des effets sur la mise en place du patron embryonnaire selon l'axe dorso-ventral mes expériences

révèlent que la perte de fonction de FrzA affecte également les mouvements cellulaires.

Une analyse fine de l'effet de la perte de fonction FrzA sur les mouvements de convergence a été réalisé et j'ai pu mettre en evidence un retard significatif dans la convergence des cellules vers la face dorsale de l'embryon. Ceci peut être du à un effet direct sur les mouvements ou à un effet indirect qui impliquerait les effets de FrzA sur l'activité de la voie de signalisation BMP, peut être via la surexpression de chordin.

4 - Similarité et différence entre FrzA et les autre inhibiteur de Wnt: FrzB et Dkk1

a. Analyse comparative des phénotypes de perte de fonction de FrzA, FrzB et DKK1

FrzA et FrzB appartiennent tous deux à la même famille des SFRP. Le mode d'action de FrzB étant connu, j'ai comparé les activités de ces deux gènes ainsi que celle de DKK1 qui controle également l'activité de Wnt8 par un autre mécanisme, au niveau des phénotypes générés par leur perte de fonction. De manière intéressante, dans chacun des cas, bien qu'avec des nuances, un phénotype de dorsalisation associé à une surexpression de chordin a été observé.

b. Sauvetage par perte de fonction de chordin ou gain de fonction de BMP des effets générés par la perte de fonction FrzA, FrzB et DKK1

Les phénotypes comparés ayant montré que chordin était surexprimé chez les embryos perte de fonction pour ces trois facteurs j'ai tenté de sauver ces phénotypes via une perte de fonction de chordin ou une surexpression de

BMP. J'ai ainsi pu constater un sauvetage de certains au moins des éléments phénotypiques par une activation de da voie BMP our une perte d'activité de son inhibiteur chordin. De ce point de vue au moins ces trois facteurs semblent fonctionner de manière semblable.

c. Analyse comparative des phénotypes de surexpression de FrzA, FrzB et DKK1

Une approche comparative similaire a été utilisée pour les phénotypes de gain de fonction par surexpression de ces trois facteurs. Dans ce cas, à l'opposé de ce que j'ai pu observer pour la perte de fonction FrzA et FrzB/DKK1 semblent fonctionner de manière différente et alors que la surexpression de FrzB/DKK1 mime la perte de fonction de Wnt8 et conduit à une surexpression de chordin dans toute la région marginale, la surexpression de FrzA génère une perte de fonction de chordin ce qui suggère fortement que FrzA est incapable d'inhiber l'activité zygotique de Wnt8.

d. Sauvetage des phénotypes de perte de fonction de FrzA par la surexpression de FrzB et DKK1

Un argument supplémentaire prouvant le lien différent entre FrzA et FrzB/DKK1 pour l'activité Wnt8 vient de ce que la perte de fonction de FrzA ne peut pas être sauvé par l'injection d'ARN codant pour FrzB ou DKK1 alors que son propre ARN sauve totalement le phénotype des morphants. Il est donc clair que FrzA et FrzB/DKK1 ont des modes d'action différents, conclusion qui s'oppose aux premières observations suite à la comparaison des phénotypes de perte de fonction de ces trois gènes.

5 - Interaction entre FrzA et la voie de signalisation Wnt8 zygotique

a. Sauvetage de surexpression Wnt8 par co-injection de FrzA

Bien que FrzA agisse de manière différente à FrzB et DKK, j'ai pu montrer que la surexpression de FrzA peut sauver les phénotypes de posteriorisation induits par une surexpression de Wnt8 via l'injection d'un plasmide surexprimant ce gène (activité Wnt8 zygotique).

b. Sauvetage de phénotype de perte de fonction FrzA par inhibition de Wnt8

Si FrzA bloque directement l'activité de Wnt8, alors une perte de fonction de FrzA devrait résulter en une augmentation de l'activité de Wnt8. J'ai donc fait l'hypothèse qu'une perte de fonction de Wnt8 devrait sauver en partie au moins la perte de fonction de FrzA. Cependant j'ai infirmé cette hypothèse en mettant en évidence un accroissement de l'activité ectopique de chordin apparaissant chez les morphants FrzA lorsque Wnt8 était inhibé conjointement.

c. Résumé des interactions entre Wnt8 et FrzA:

L'ensemble des expériences réalisées par gain et perte de fonction de Wnt8 et de FrzA ont conduit à des résultats contradictoires. Cependant de nombreuses données suggèrent que Wnt8 ne soit pas la cible directe de FrzA.

6. Mecanisme d'action de FrzA pour la repression de chordin:

a. FrzA agit à courte distance

Les territoires d'expression de FrzA et de Wnt8 étant mutuellement exclusifs, l'un exprimé à la marge (Wnt8) et l'autre en étant exclu, j'ai fait l'hypothèse que FrzA ne diffusait pas dans la region marginale (puisque mes données précédentes montrent que sa surexpression peut bloquer l'activité de Wnt8). J'ai marqué la protéine FrzA et dans un test réalisé en culture de cellules j'ai pu montrer que FrzA ne pouvait pas être détecté dans le milieu de culture et n'était présent que dans ou associé au cellules. Sur la base de ces expériences je peux conclure que FrzA agit à courte distance, vraisemblablement de manière paracrine.

b. FrzA inhibe l'expression de Chordin en interférant avec l'activité Wnt maternelle.

Les données ci-dessus suggèrent que FrzA agisse non pas sur le contrôle de l'activité Wnt8 zygotique mais puisse contrôler l'activité maternelle (et ceci est en accord avec l'expression maternelle de FrzA). J'ai pu montrer que l'absence de FrzA conduit à une activation de la voie de signalisation Wnt maternelle et que cette activation peut être bloquée par un répresseur spécifique de l'activité maternelle de Wnt. De plus en utilisant des formes tronquées de la b-caténine j'ai montré que FrzA agit en amont de le phosphorylation de la b-caténine dans la régulation de l'activité maternelle de Wnt.

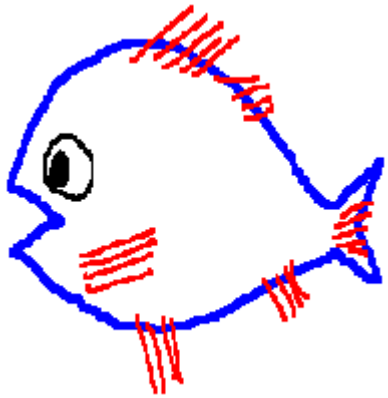
7. FrzA agit au niveau du récepteur Frizzled 2:

Par des expériences de perte de fonction de Frzd2 j'ai pu montrer que FrzA agit en amont de Frzd2 et que ce dernier est vraisemblablement le médiateur principal de l'activité FrzA. J'ai ensuite montré que l'inhibition de la

signalisation Wnt maternelle par FrzA dépend de Frzd2. Comme FrzA contient un domaine CRD il se peut qu'il se lie directement à Frzd2.

J'ai testé l'hypothèse d'une interaction directe de Frzd2 et de FrzA in vitro. J'ai pu montrer qu'in vitro FrzA est capable de se lier à Frzd2 mais également à Wnt8 et Wnt11 ce qui est en accord avec les observations précédentes concernant SFRP1 dans d'autres espèces. J'ai ensuite fait l'hypothèse que les phénotypes de surexpression de FrzA résultait de l'interaction avec Frzd2, par exemple en empêchant ce dernier de dimériser, ou en formant un complexe Frzd2-FrzA inactif. Les différentes expériences de gain et perte de fonction de Frzd2 et FrzA en vue de tester cette hypothèse ne m'ont cependant pas permis d'obtenir un resultat décisif et d'autres expériences devront être menées pour résoudre cette question.

Part I
General introduction



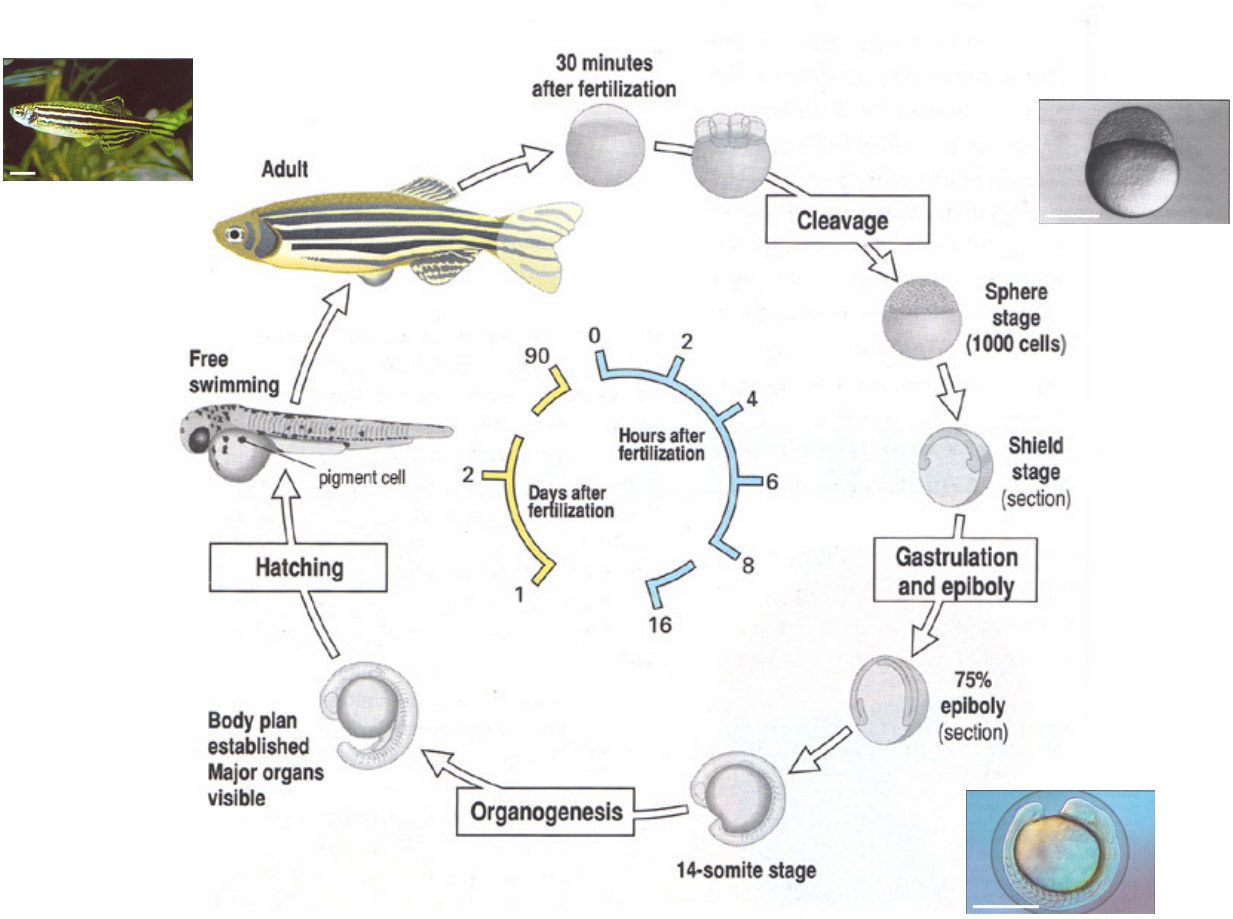


Figure I-1.: Life cycle of the zebrafish (photographs from Wolpert, et al., 2002 in Principles of Development, Oxford press). The zebrafish embryo develops as a cup-shaped blastoderm sitting on top of a large yolk cell. It develops rapidly and by 2 days after fertilization the tiny fish, still attached to the remains of its yolk, hatches out of the egg. The up-right side paragraph shows a zebrafish embryo at the sphere stage of development, with the embryo sitting on top of the large yolk cell (scale bar = 0.5 mm). The low-right side paragraph shows an embryo at the 14-somite stage, showing developing organ systems. Its transparency is useful for observing cell behavior (scale bar =0.5 mm). The upper-left side paragraph shows an adult zebrafish (scale bar = 1 cm). Paragraphs of sphere stage from C. Kimmel, 14-somites stages from N. Holder, adult stage from M. Westerfield.

A. The zebrafish - a useful vertebrate model

The zebrafish is originally from Southeast Asia and is a tropical fish. It has become a powerful tool for studying developmental mechanisms in vertebrates. There are several advantages with using the zebrafish to conduct various experiments. The embryos are relatively large, about 0.7 mm in diameter, and its development can be easily observed through the chorion. Its development is very rapid, all common vertebrate-specific body features can be seen at around 2 days, and it is transparent in the early developmental stage during the first 24 hours (Fig. I-1). As a result, this allows observation of developing organs including the compartmentalized brain, eyes, ears and all internal organs. Being transparent also allows researchers to identify the fate of individual cells during development (Kimmel and Law, 1985a; Kimmel and Law, 1985b; Kimmel and Law, 1985c).

In addition, fertilization and subsequent embryonic development are external and occur synchronously in large clutches. Although the generation time is not particular short (2~4 months) compared to other vertebrates, the large progeny still make it suitable for large-scale screening, including genetic and small-molecule drug screens.

Research on the zebrafish began with George Streisinger who first exploited the possibilities of genetically manipulating this species, including *in vitro* fertilization, a method to induce haploid development, and establishment of homozygous lines (Streisinger et al., 1981). In 1996, Nusslein-Volhard's and Wolfgang Driver's groups conducted large-scale screening which resulted in nearly 2000 characterized mutants by high-efficiency *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis methods (Development Issue 123, 1996). Currently, the zebrafish is being used for various projects related to humans including

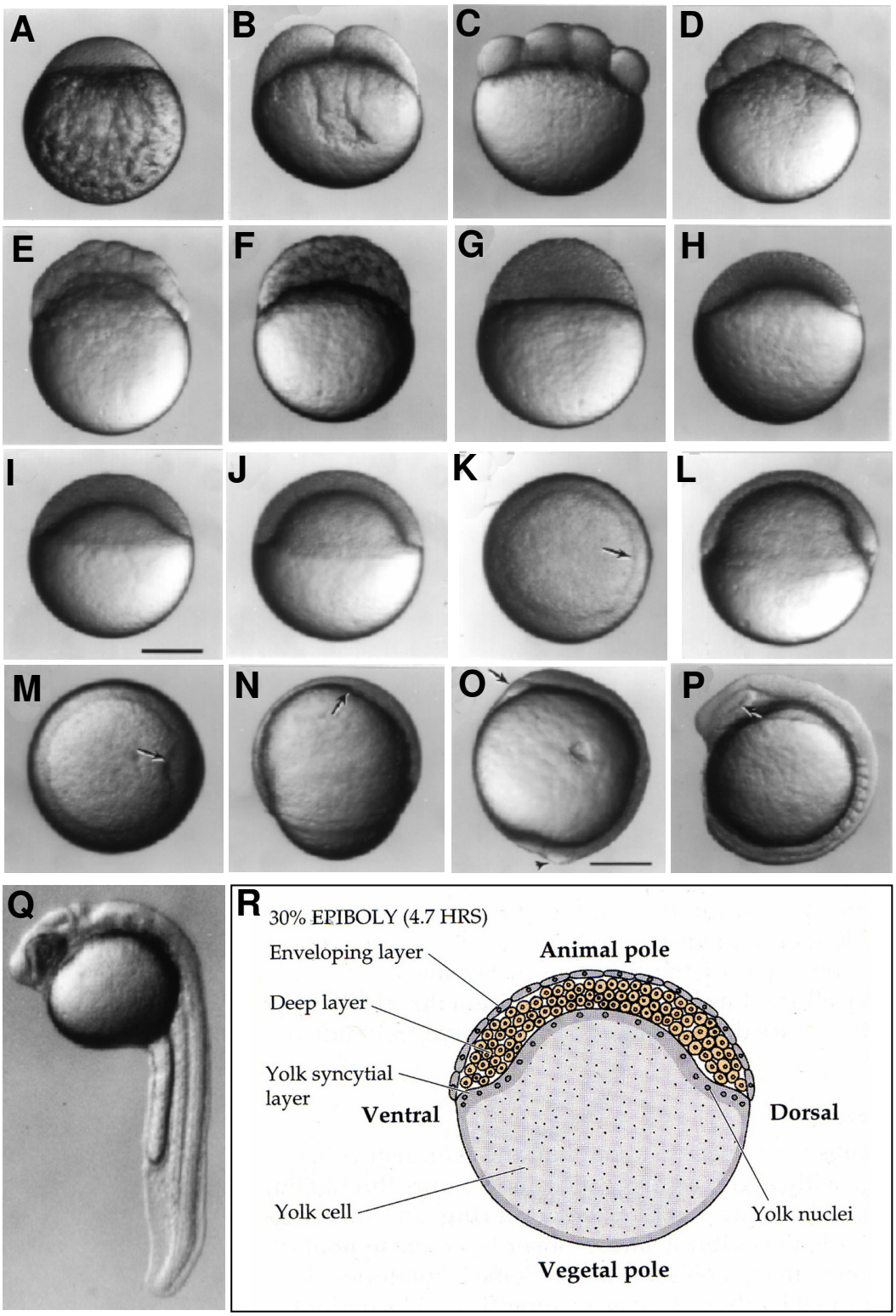


Figure I-2 : The different developmental stage of Zebrafish (photographs from Kimmel et al., 1995 and Langeland and Kimmel, 1997). Zygotic stage (A). Cleavage stage includes 2 cells (B), 8 cells (C), 32 cells (D), 64 cells (E). Blastula stage includes 128 cells (F), 512 (G), Dome (H), 30 % epiboly (I). Gastrula stage includes 50 % epiboly (J), Germ ring (K), Shield (L, M), 70 % epiboly (N), Tail bud (O). Somitogenesis stage (P) and 24 hours stage (Q). Figure (R) illustrates detail structure of the 30% epiboly. Arrows indicate ring (K), shield (M), internalization terminals (N), anteriorposterior terminal (O), optic premordium (P). The scale bar indicate 250 µm. All the embryos are lateral view except (K) is animal pole view.

inflammation, angiogenesis, cancer drug design, neuronal growth, and bone and muscle formation, and has become an increasingly popular model.

B. Early development of zebrafish embryos

The newly fertilized egg remains in the zygote period (for around 40 min, Fig. I-2A) until the cleavage stage.

In the cleavage stage, the zygote undergoes cleavage in a mound of blastomeres perched above the yolk (Fig. I-2B-D). The first five cleavages are all vertical, and the first horizontal cleavage occurs when the cleavage gives rise to the 32-cell stage (Fig. I-2E).

Further cleavage of the blastoderm gives rise to formation of a single outer layer called the outer enveloping layer (EVL) and a deep layer of more-rounded cells (DEL) which overlay the yolk (Fig. I-2F-I, R). At this stage, the embryo enters the blastula period because the blastodisc begins to look ball-like at the 128-cell stage until the time of gastrulation. At this stage, cells divide synchronously in the beginning, but began lengthening in the mid-blastula stage (MBT, (Kane and Kimmel, 1993), Fig. I-2G) at the 512-cell stage and the tenth cell division cycle. In the MBT, RNA synthesis increases over the background level, and this is considered the beginning of zygotic gene transcription (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b). Before zygotic gene expression occurs, there are many maternal RNAs and proteins which are responsible for early development (Pelegri, 2003). Just after the MBT, the yolk syncytial layer (YSL), which is unique in teleosts, forms, and it is an extraembryonic layer that arises from marginal blastomeres, which during the early blastula stage, collapse onto the yolk cell, forming a ring around the blastodisc edge (Fig. I-2R). The blastoderm expands in the vegetal direction in the late blastula stage by a spreading process known

as epiboly to cover the yolk. During this stage, the blastodisc thins considerably, changing from a high-piled cell mound to a cup-shaped cell multilayer of nearly uniform thickness (Fig. I-2I). This change accompanies the radical intercalation which is the earliest cell movement. The marginal deep blastomeres show less mixing compare to central deep blastomeres, and this is considered an important reason for the formation of the early specification of mesoderm identities (Kimmel et al., 1991).

The embryos then go into the next stage, the gastrula stage (Fig. I-2J-O). In the gastrula stage, epiboly continues until the margin of the blastoderm advances around the yolk cell to completely cover it, and several cell movements are included in this stage: internalization, convergence, and extension. The beginning of internalization is considered the beginning of the gastrulation stage. When internalization begins (around 5.7 h post-fertilization (hpf)), the germ ring becomes visible and simultaneously almost completely surrounds the blastoderm rim (Fig. I-2K). Soon thereafter, convergence begins and produces a local accumulation of cells which is called the embryo shield and becomes the future dorsal side (Fig. I-2M). At this stage, the future dorsal-ventral (DV) and anterior-posterior (AP) sides can clearly be identified. The epiboly is temporarily arrested while forming the shield, but begins again when the shield is finished. The margin of the blastoderm advances around the yolk cell to completely cover it at the tail bud stage (Fig. I-2O). When the internalization movement occurs, DEL (deep cell layer) cells internalize, become the hypoblast, and separate into the epiblast which is the upper layer of the cell. After epiboly is complete, only the ectoderm (including the neural and epidermal) is left of the entire epiblast. The internalized hypoblast layer becomes the mesoderm and endoderm. At the same time, the dorsal converges from the lateral region, and the dorsal blastoderm thickens due to

mediolateral intercalation (Keller and Tibbetts, 1989), which is composed of both the epiblast and hypoblast layers of cells on the dorsal side (Fig. I-2N). Mediolateral intercalation is the force that extends the primary axis length. At about 90% epiboly, the axial and paraxial hypoblasts become distinct.

After gastrulation, the embryo enters the segmentation stage (10~24 hpf, Fig. I-2P). The most obvious phenotype is the formation of the somites from the anterior to posterior (AP) at three per hour in the first 6 hour and two per hour thereafter. The body length also obviously increases at this time, and the first body movements appear. The extended axis (on the dorsal side) curls around the original vegetal pole and towards the head but strengthens in the later segmentation stage. The endoderm also becomes increasingly morphologically distinctive at this stage. The neural ectoderm undergoes extensive morphogenesis at this stage including thickening of the neural plate especially on the anterior region due to cells becoming columnar shaped, infolding at the midline, development into the neural keel, and formation of the anterior brain rudiment which eventually develops into the forebrain (telencephalon and diencephalon) and midbrain (mesencephalon). The rudiment of the eyes (at the nine-somite stage) and the optic primordia can also be distinguished at this stage. The formation of other neural primordia, including the hypothalamus, epiphysis, optic tectum, rhombomeres (r1~r7), neural crest cells, otic primordium, and primary neurons, is also established at this stage.

In the subsequent pharyngula stage (24~48 hpf, Fig. I-2Q), the embryo formats more-complete structures like the notochord, five lobes of the brain, and pharyngeal arches. Pigment cells appear, the circulatory system forms, and marked tactile sensitivity appears. After this stage, the embryo hatches, begins to swim, and becomes a larva.

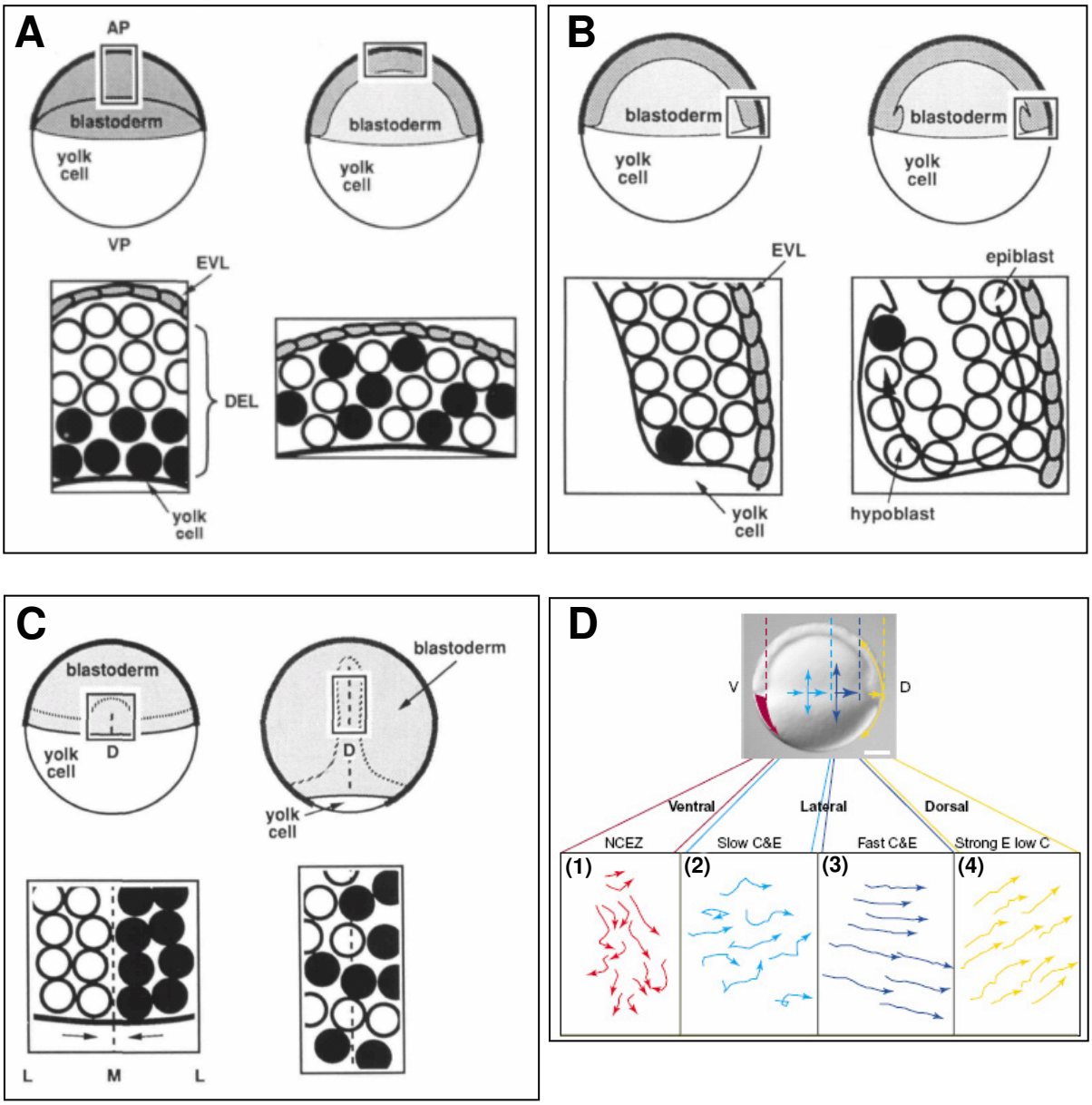


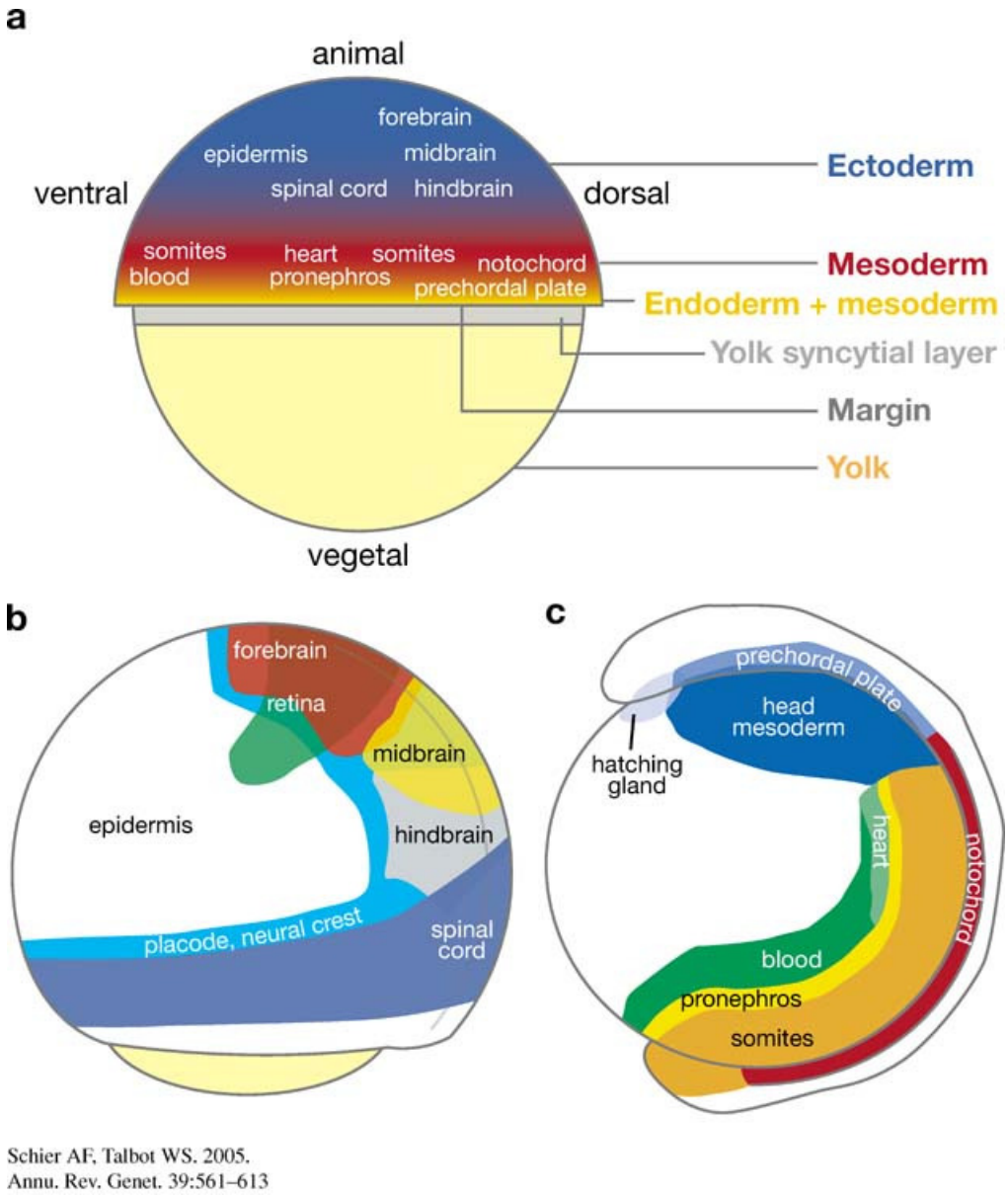
Figure I-3: Cut-away diagram of the different cell movements on the different developmental stages in the zebrafish embryo (photographs from Warga and Kimmel, 1990 and Myers et al., 2002). (A) The Radial intercalation movement that happened between the sphere (Left) and until the gastrulation stage (Right). Deep DEL cells move outward (radially), intercalating among more superfacial DEL cells but not among EVL cells This movement contributes to epiboly, thinning and spreading the blastoderm. (B) The involution movement that happened between the 50 % epiboly (Left) and the ring stage (Right). A DEL cell first at the blastoderm margin (labeled by black) is at the front of the wave of internalizating cells. This movement generates the hypoblast. EVL cells do not move. (C) The mediolateral intercalations that showed at the shield stage (Left) and the 80% epiboly stage (Right). DEL cells converge towards the dorsal midline, moving from lateral to medial positions. This movement lengthens the embryonic axis (dashed line). (D) The lateral convergence movement that illustrated with the trajectories at the shield stage. Movement trajectories were derived from time-lapse analyses at late gastrula stages. (1) Cells in the no convergence no extension zone (NCEZ, red) travel along, zig-zaging paths towards the future tailbud. Their net dorsal speed is negligible. (2) Lateral cells undergoing slow C&E meander considerably and therefore have a slow net dorsal speed. (3) Lateral cells engaged in fast C&E move along relatively straight trajectories, resulting in an increased net dorsal speed. (4) The paths of dorsal cells are oriented dorsally and biased towards anterior or posterior, depending on the position of the cell along the animal-vegetal axis, their net dorsal speed is reduced compared with lateral cells participating in fast C&E movements. Scale bar, 100 μm. Abbreviations: D, dorsal; V, ventral. All the embryos are from the lateral view except the (C) are from dorsal view.

C. Different cell movements during zebrafish early development

The first cell movement is the radial intercalations which occur in the beginning of epiboly formation. This is accomplished by the deepest blastomeres streaming outward, toward the surface (Fig. I-3A). As they move, they mix fairly indiscriminately among more-superficial cells along the way. Active cell repacking by these radial intercalations may be a part of the driving force of early epiboly.

Epiboly continues to move in the vegetal direction until the ring stage when another cell movement appears, internalization. Internalization produces the germ ring by folding the blastoderm back upon itself, after which the ring forms (Fig. 3B). Recent evidence showed that this cell movement does not occur by involution movement but by ingression, the inward movement of individual cells. Results showed that cells move coherently toward the margin, where they begin to form protrusions, lose coherence with their neighbors, and ingress. This “flow of individuals” results in internalization, a new term that describes this cell movement to separate it from “involution” (Rohde and Heisenberg, 2007; Schier and Talbot, 2005; Solnica-Krezel, 2006).

Soon after internalization, dorsal side toward lateral-side cells movements occurs. This so-called convergence is mediolateral movement and contributes to the cell mediolateral intercalation which is considered to be the major force promoting dorsal axis extension movement and contributing to prechordal plate migration (Fig. I-3C). The convergence movement narrows the germ layers mediolaterally, whereas extension movement lengthens them anteroposteriorly, thereby defining the dorsoventral and rostrocaudal embryonic axes (Myers et al., 2002b). The speeds of convergence and



Schier AF, Talbot WS. 2005.
Annu. Rev. Genet. 39:561–613

Figure I-4: Zebrafish fate maps from Schier and Talbot, 2005. (a) Fate map at 50% epiboly stage, the onset of gastrulation. Lateral view, dorsal to the right, animal pole to the top. Germ layers are arranged along the animal-vegetal axis. Different mesodermal and ectodermal fates are arranged along the dorsal-ventral axis. No precise boundaries are depicted because cell fates are often intermingled. (b) Fate map of ectoderm at 90% epiboly. Lateral view, dorsal to the right, animal pole and anterior to the top. (c) Model fate map of mesoderm at early somite stage. Lateral view, dorsal to the right, animal pole and anterior to the top. Note that no precise fate map has been established at this stage. Therefore, regions shown here are approximations derived in part from the expression patterns of marker genes (ZFIN. org). The posterior region of the tail bud will continue to extend and give rise to different mesodermal and ectodermal fates.

extension greatly differ depending on the location of cells. According to the time-lapse analysis on individual cells at the mid-gastrulation stage, mesodermal cell movements in different regions can be determined (Fig. I-3D). The ventral-most region cells (20°~30° arc) undergo no convergence or extension movements (NCEZ) and only engage in epibolic movement in the vegetal direction. Lateral mesodermal cell populations initially show an amoeboid morphology and take indirect, dorsal-oriented zigzagging paths, achieving a slow net dorsal speed. As they move dorsally, their trajectories become more direct, and, consequently, the net dorsal speed increases. However, these converging lateral cell populations also undergo progressively faster extension. Labelled dorsal cells exhibit strong extension with little convergence.

D. The fate map of zebrafish in the early developmental stage

The future fate map of a zebrafish embryo can be identified at the shield stage (Fig. I-4). The fate map is made by injecting single cells with lineage-tracer dye, later looking for the labelled descendants of the injected cell into various organs. Individual cells at the shield stage can commit to a particular fate. Precursors of different germ layers are arranged along the animal-vegetal axis. The ectoderm is located animally, the mesoderm is located more marginally, and the endoderm, intermingled with the mesoderm, is located in the most marginal position (Fig I-4a, (Schier and Talbot, 2005)). Because specific cell fates can be identified at this stage, in many experiments including mine, different markers can be used to label each group of cells in order to investigate early pattern changes. The non-neural ectoderm (epidermis labelled by Foxi 1) is derived from the animal-ventral territory. The

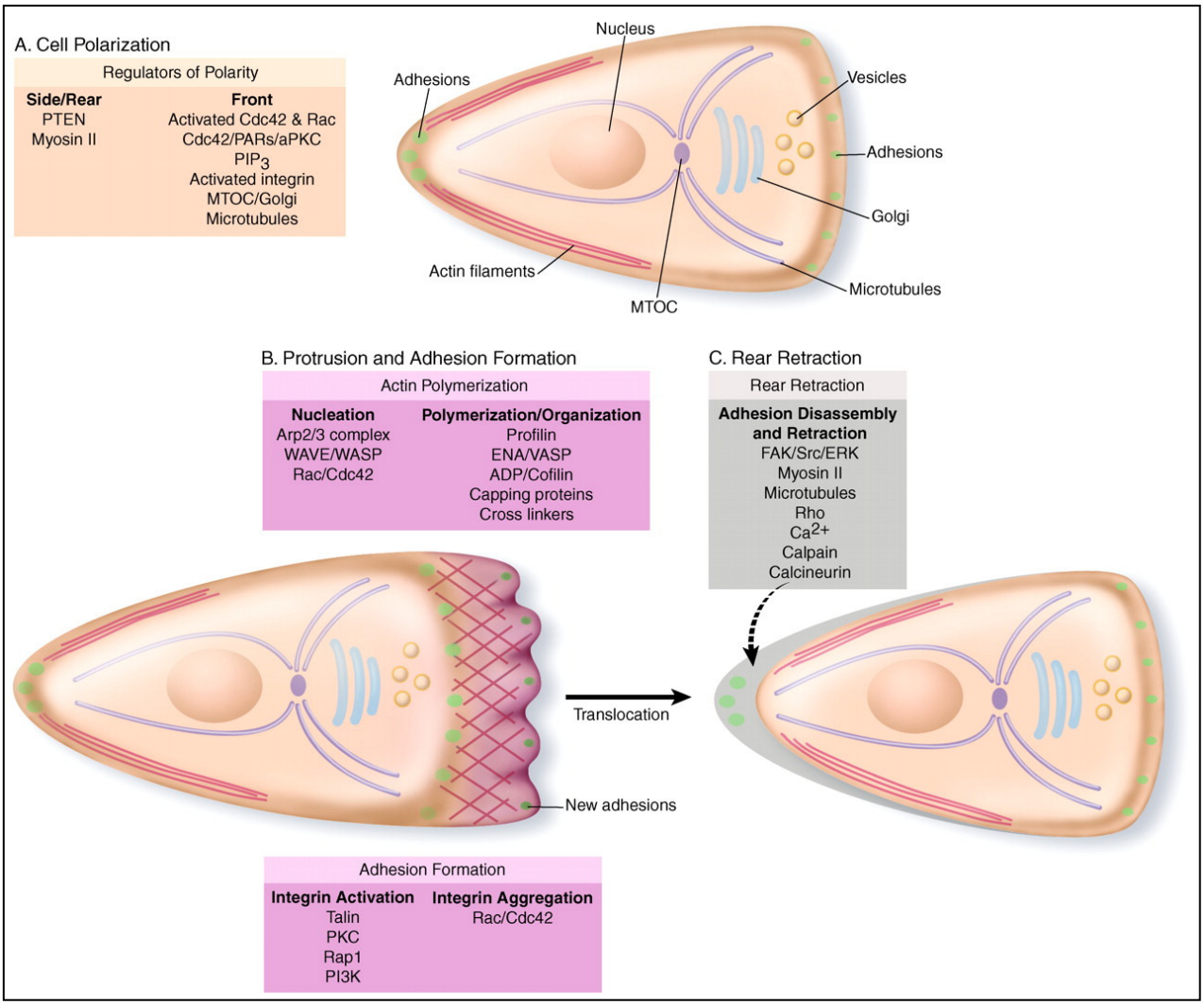


Figure I-5: Steps in cell migration (photograph from Ridley et al., 2003). Polarity is intrinsic to a migrating cell (**A**).Cdc42, along with Par proteins and aPKC, are involved in the generation of polarity. Several additional proteins are implicated in polarity, which results in directed vesicle trafficking toward the leading edge, organization of microtubules (in some cells), and the localization of the MTOC (in some cells) and Golgi apparatus in front of the nucleus. In the presence of a chemotactic agent, PIP3 is produced at the leading edge through the localized action of PI3K, which resides at the leading edge, and PTEN, a PIP3 phosphatase that resides at the cell margins and rear. PTEN and myosin II are implicated in restricting protrusions to the cell front. The migration cycle begins with the formation of a protrusion (**B**). WASP/WAVE proteins are targets of Rac and Cdc42 and other signaling pathways and regulate the formation of actin branches on existing actin filaments by their action on the Arp2/3 complex. Actin polymerization, in turn, is regulated by proteins that control the availability of activated actin monomers (profilin) and debranching and depolymerizing proteins (ADF/cofilin), as well as capping and severing proteins. Protrusions are stabilized by the formation of adhesions. This process requires integrin activation, clustering, and the recruitment of structural and signaling components to nascent adhesions. Integrins are activated by talin binding and through PKC-, Rap1-, and PI3K-mediated pathways. Integrin clustering results from binding to multivalent ligands and is regulated by Rac. At the cell rear, adhesions disassemble as the rear retracts (**C**). This process is mediated by several possibly related signaling pathways that include Src/FAK/ERK, Rho, myosin II, calcium, calcineurin, calpain, and the delivery of components by microtubules. Many of these molecules may also regulate the disassembly of adhesions at the cell front, behind the leading edge.

forebrain and midbrain progenitors are found animally and dorsally (Fig. I-4b), whereas hindbrain and spinal cord precursors are located closer to the margin and more laterally and ventrally, respectively. Precursors of the different mesodermal cell types are arranged along the dorsal-ventral direction with the dorsal corresponding to the side of the shield. Mesoderm cells located the most dorsally give rise to the notochord and prechordal plate (at the 70% epiboly stage, Fig. I-4C). More-lateral cells give rise to trunk somites and the heart. Blood and pronephros are derived from marginal blastomeres that are closest to the ventral region. Different endodermal precursors are also distributed along the dorsal-ventral direction. The pharynx is located most dorsally, while the stomach, intestines, and liver are located more laterally and ventrally. Because of complex gastrula movements that include involution of the hypoblast from the blastomere margin to the animal pole direction, the cell fate predicted should be combined with the 3D cell movement effect. For example, the forebrain and prechordal plate form on the most anterior region of the head and lie at opposite positions of the animal-vegetal axis at the onset of gastrulation.

E. Proteins linked to cell movement or cell fate determination

1. Proteins linked to cell movement

a. Molecular mechanisms of cell migration

Cell migration can be conceptualized as a cyclic process (Fig. I-5). In the first part when a cell responds to a migration-promoting agent, the cell becomes polarized and extends protrusions in the direction of migration. The protrusions, which format the lamellipodia or filopodia, are usually driven by actin polymerization, and are stabilized by adhering to the extracellular matrix

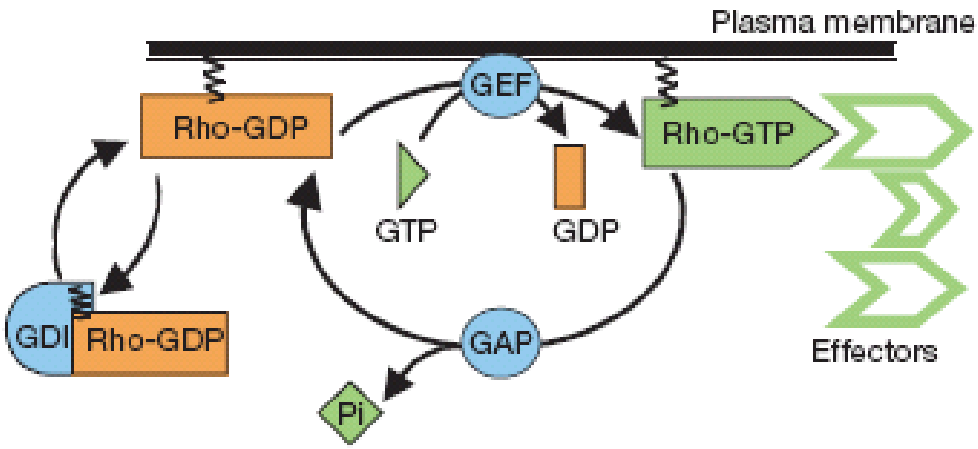


Figure I-6: The Rho GTPase cycle (photograph orm Etienne-Manneville and Hall, 2002). They cycle between an active (GTP-bound) and an inactive (GDP-bound) conformation. In the active state, they interact with one of over 60 target proteins (effectors). The cycle is highly regulated by three classes of protein: in mammalian cells, around 60 guanine nucleotide exchange factors (GEFs) catalyze nucleotide exchange and mediate activation; more than 70 GTPase-activating proteins (GAPs) stimulate GTP hydrolysis, leading to inactivation; and four guanine nucleotide exchange inhibitors (GDIs) extract the inactive GTPase from membranes.

(ECM) or adjacent cells via transmembrane receptors linked to the actin cytoskeleton. The second part is adhesion between migrating cells and adjacent cells which serves as a traction site for migration as the cell moves forward over them. The third part consists of the rear of the migrating cell disassembling so that it can be dispatched (Ridley et al., 2003).

Establishment and maintenance of cell polarity are mediated by a set of interlinked positive feedback loops involving Rho family GTPases, phosphoinositide 3-kinases (PI3Ks), integrins, microtubules, and vesicular transport (Fig. I-5A). Rho family GTPases are guanine nucleotide (GTP)-binding proteins consisting of the Rho, Rac, and Cdc42 subfamilies. The proteins exist in two interconvertible forms: GDP-bound inactive and GTP-bound active forms. Active GTPases interact with specific downstream targets and perform their cellular functions. The GTP-bound active form becomes inactive after GTP hydrolysis and liberation of phosphate. GTP-GDP exchange reactions are regulated by guanine nucleotide exchange factors (GEFs), guanine nucleotide dissociation inhibitors (GDIs), and GTPase-activating proteins (GAPs) (Fig. I-6). The major GTPase in the Rho GTPase family that controls cell polarity is Cdc42. Cdc42 is active toward the front of migrating cells (Itoh et al., 2002), and both inhibition and global activation of Cdc42 can disrupt the directionality of migration (Etienne-Manneville and Hall, 2002). Cdc42 affects the polarity by localizing the microtubule-organizing center (MTOC) and Golgi apparatus in front of the nucleus, orienting them toward the leading edge. This orientation may contribute to microtubule-mediated delivery of Golgi-derived vesicles to the leading edge, providing the associated proteins needed for forward protrusion (Etienne-Manneville and Hall, 2002; Rodriguez et al., 2003). The effect of Cdc42 on the MTOC position is mediated by a complex which includes PARs

and atypical protein kinase C (aPKC) (Etienne-Manneville and Hall, 2003b). The downstream target of Cdc42, the kinase, PAK1, can itself mediate Cdc42 activation downstream of heterotrimeric GTP-binding protein (G protein)-coupled receptors. These interactions define a positive feedback loop between Cdc42 and PAK1, resulting in high Cdc42 activity at the leading edge (Li et al., 2003). Other feedback loops involving integrins may also contribute to maintaining local Cdc42 activation (Etienne-Manneville and Hall, 2001; Etienne-Manneville and Hall, 2002). In addition, there is another feedback loop which includes Cdc42, PI3Ks, and PTEN that work together to initiate and maintain cell polarity. When a chemoattractant appears, PIP_3 and $PI(3,4)P_2$ generated by PI3Ks become rapidly and highly polarized in cells. This polarized situation can also be generated by the phosphatase, PTEN, that can inactivate them. As a result, when cells become polarized, PI3Ks accumulate on the leading edge, whereas PTEN becomes restricted to the sides and rear (Devreotes and Janetopoulos, 2003; Merlot and Firtel, 2003). In addition, Cdc42 activation is implicated in PTEN exclusion, and PIP3 is required for localizing Cdc42 activity (Li et al., 2003) (Fig. I-5A).

Actin polymerization provides the major polarity for protrusion formation (Fig. I-5B). Several actin-binding proteins regulate the rate and organization of protrusions by affecting the pool of available monomers and free ends (dos Remedios et al., 2003; Pollard and Borisy, 2003). Profilin prevents self-nucleation by binding to actin monomers and also selectively targets monomers to the leading edge. Ena/VASP proteins which bind the leading edge of actin filaments are enriched in filopidial tips and allow continuing elongation of filaments. The Rho GTPase family which includes Rac and Cdc42 also plays a major role in initiating protrusion and actin polymerization. The major target of Rac and Cdc42 that mediates actin polymerization in

protrusions is the WASP/WAVE family of Arp2/3 complex activators. Rac stimulates lamellipodial extension by activating WAVE proteins (Cory and Ridley, 2002). Cdc42 binds to WASP proteins and stimulates the Arp2/3 complex to induce dendritic actin polymerization (Welch and Mullins, 2002). WAVE/WASP can regulate Rac and Cdc42 activities by binding to GAPs and GEFs (Cory et al., 2002; Hussain et al., 2001; Soderling et al., 2002). Therefore, this might generate a positive feedback loop for actin polymerization.

When cells begin to migrate, integrin serves as a major family of migration-promoting receptors (Fig. I-5B). Integrins are heterodimeric receptors consisting of α and β chains with large ligand-binding domains and short cytoplasmic domains. The binding of ligands to the extracellular domain leads to conformational changes in the receptors by changing interactions between the α- and β-chain cytoplasmic domains (Emsley et al., 2000) and to integrin clustering. This change initiates intracellular signalling including activation of small GTPase during migration (Geiger et al., 2001). The activated integrin preferentially localizes to the newly formed leading edge (Kiosses et al., 2001). Integrin affinity is regulated in large part by alterations in the conformation of integrin extracellular domains that result from interactions at the integrin cytoplasmic tail (Kim et al., 2003). Activation of key intermediates such as the GTPases, Rap1 or PKC can also increase integrin affinity. The cytoskeletal linker protein, talin, promotes integrin activation by binding to a subset of integrin β-subunit tails and disrupting integrin α-β-subunit tail interactions (Kim et al., 2003; Tadokoro et al., 2003).

When cells finish migrating, adhesion should be disassembled both at the front and rear of cells (Fig. I-5C). Adhesion disassembly at the leading edge is little known compared to maturation. Evidence shows that protein kinase and

phosphatase including FAK, Src, and ERK play central roles in adhesion turnover and stability (Alahari et al., 2002; Brahmbhatt and Klemke, 2003; Larsen et al., 2003; Turner et al., 2001; Webb et al., 2002). For adhesion disassembly in the rear, the high tension exerted on the rear adhesions contributes to detachment (Cotran et al., 1999). Several lines of evidence point to a role for myosin II in this event as well as in the maintenance of polarity. Myosin II assembly can be blocked by inhibiting Rho GTPase or Rho kinase and impairing retraction (Worthylake and Burridge, 2003; Xu et al., 2003). Molecules that regulate front leading edge turnover include FAK and Src which also work at the rear. In addition, intracellular calcium levels are implicated in the disassembly of adhesion in the rear. Potential targets for calcium such as the calcium-regulated phosphatase, calcineurin, and calcium-activated protease, calpain, which is also activated by ERK, have the potential to cleave several focal adhesion proteins, including integrins, talin, vinculin, and FAK (Glading et al., 2002; Hendey et al., 1992).

b. Molecules that control cell movement in zebrafish embryos

(1) Molecules that contribute to epiboly formation and internalization

There are four maternal mutants (*betty boop*, *poky, slow,* and *bedazzled*) which can control epiboly formation (Wagner et al., 2004). The *betty boop* display premature constriction of the margin, which is required for progression past the 50% epiboly stage. The mutants (*poky, slow,* and *bedazzled*) show slower or delayed epiboly formation in different ways: *poky* is required for movement of all three cell layers in epiboly and *slow*'s function predominates in the deep layer. The *bedazzled* mutant displays a delayed movement of the

epiboly as well as wide separation of the EVL and DEL margins during mid-epiboly stages, indicating a more-severe effect on the DEL than the EVL. However, the functions of these four mutants only act on epiboly movement without changing the patterning. The actual molecules that control these mutants are still under investigation. E-cadherin, which controls adhesion between cells, plays an important role in epiboly formation. The mutant for E-cadherin (*half-baked*) severely affects epiboly formation and arrests the vegetal spread of deep cells during gastrulation (Kane et al., 2005; Shimizu et al., 2005b). The mutant cells can intercalate but often deintercalate into the deeper layer and do not flatten. E-cadherin antisense morpholino leads to deadhesion and disintegration of the cells already at the cleavage stage (Babb and Marrs, 2004).

The cytoskeleton, including microtubules and actins, also contributes to epiboly formation. The yolk cell is equipped with two distinct microtubule arrays (Fig. I-7A). The region populated by the yolk syncytial nuclei exhibits a dense network of microtubules from which an array of parallel microtubules, oriented along the animal-vegetal axis, extends into the nucleus-free yolk cytoplasmic layer (Solnica-Krezel and Driever, 1994). Disruption of the microtubules completely blocks vegetal movement of the YSL, but only partially prevents epiboly of the EVL and deep cells. In the external YSL, there is a massive endocytosis mechanism that is independent of microtubule function. This endocytosis removes the external YSL membrane and leads to expansion of the internal YSL membrane under the blastoderm (Fig. I-7A). Finally, the epiboly can tow the EVL with it. There are three actin structures which form when the blastoderm covers half of the yolk cell (Fig. I-7B). The first, a punctate actin band, forms in the external YSL just vegetal to the EVL margin and overlaps the process of active endocytosis. The other two are ring-like

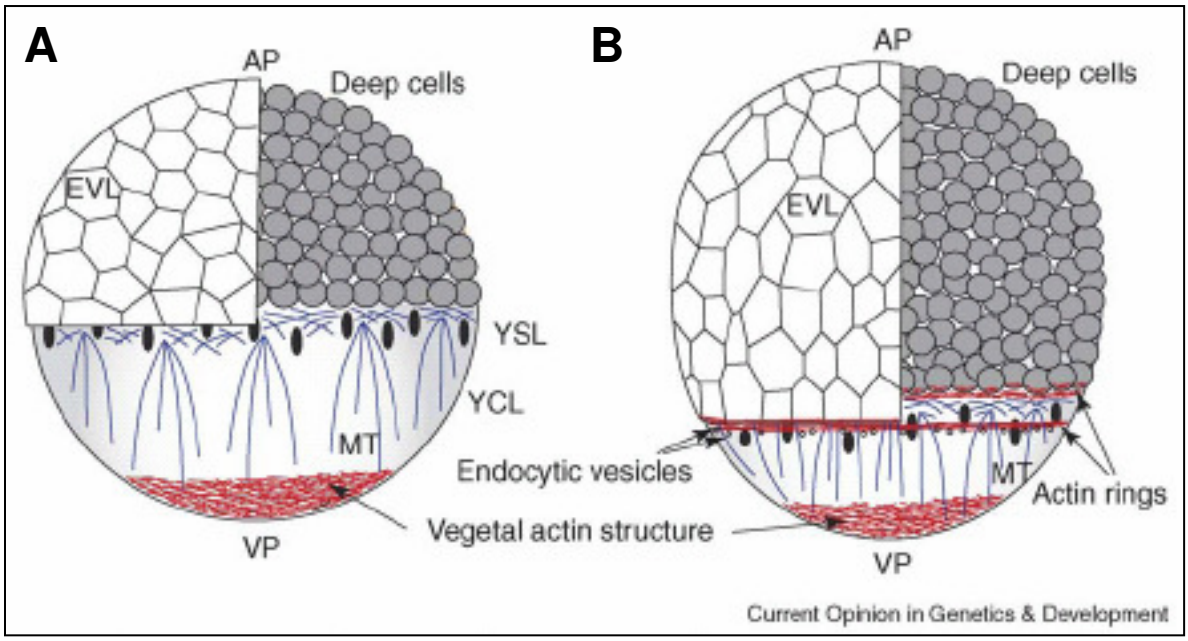


Figure I-7 : Organization of cytoskeleton in the zebrafish yolk cell (photograph from Solnica-Krezel, 2006). (A) At early gastrulation, the blastoderm and the YSL move halfway towards the vegetal pole. The enveloping layer (EVL) leads the deep cells in the epibolic movement. Within the yolk cell, a dense network of microtubules surrounds the yolk syncytial nuclei (YSN) beneath and vegetal to the blastoderm. Another set of animal–vegetal-oriented long microtubules emerges from the yolk syncytial layer into the yolk cytoplasmic layer. A dense actin structure covers the vegetal cortical region of the yolk cell. (B) At late gastrulation, the EVL moves towards the vegetal pole, in front of the deep cells. YSN lead in the epibolic movement. The network and the animal–vegetal oriented sets of microtubules are also present in the cortex of the yolk cell. Actin microfilaments continue to form a dense structure covering the vegetal cortical region of the yolk cell. Actin structures include the following: an actin ring in the vegetal margin of deep cells; an actin ring in the vegetal margin of EVL; and a punctate actin ring vegetal to the blastoderm, where endocytosis takes place. AP: animal pole, VP: vegetal pole.

structures which form at the margins of deep cells and the EVL. Inhibition of these actin structures by cytochalasin B results in a slowing down of and failure to complete epiboly (Solnica-Krezel, 2006). There is another piece of evidence showing that pregnenolone, a lipid produced from cholesterol by the Cyp11a1 enzyme in the yolk, is required to maintain a level of polymerized microtubules to ensure normal epiboly (Hsu et al., 2006).

Molecules involved in internalization are mainly on the Nodal signalling pathway. The complete absence of a Nodal signal in the *sqt;cyc* and one eye pinhead (*oep*) mutants blocks all internalization movements (Carmany-Rampey and Schier, 2001; Feldman et al., 2000). Conversely, upregulation of the Nodal pathway in the absence of the antagonist, Lefty, leads to prolonged and increased internalization (Feldman et al., 2002). The same move can also be determined by transplanting a single cell. When a single cell that lacks Nodal signalling was placed in the wild-type margin, the cell was initially internalized but then egressed (Carmany-Rampey and Schier, 2001). In contrast, if the cell is activated in the Nodal pathway and then transplanted to the Nodal pathway mutant environment, the cell can still ingress and expresses the mesendodermal marker. These data indicate the importance of Nodal signalling in the internalization movement.

(2) Molecules that contribute to convergence extension movement

Much intense work has been performed to identify molecules that involved in convergence and convergence movements. The pathway with more-detailed research is the PCP (planar cell polarity) pathway which was initially determined in *Drosophila*. Signalling molecules in vertebrates can be described from outside the cell into the Frizzled receptor and then into several

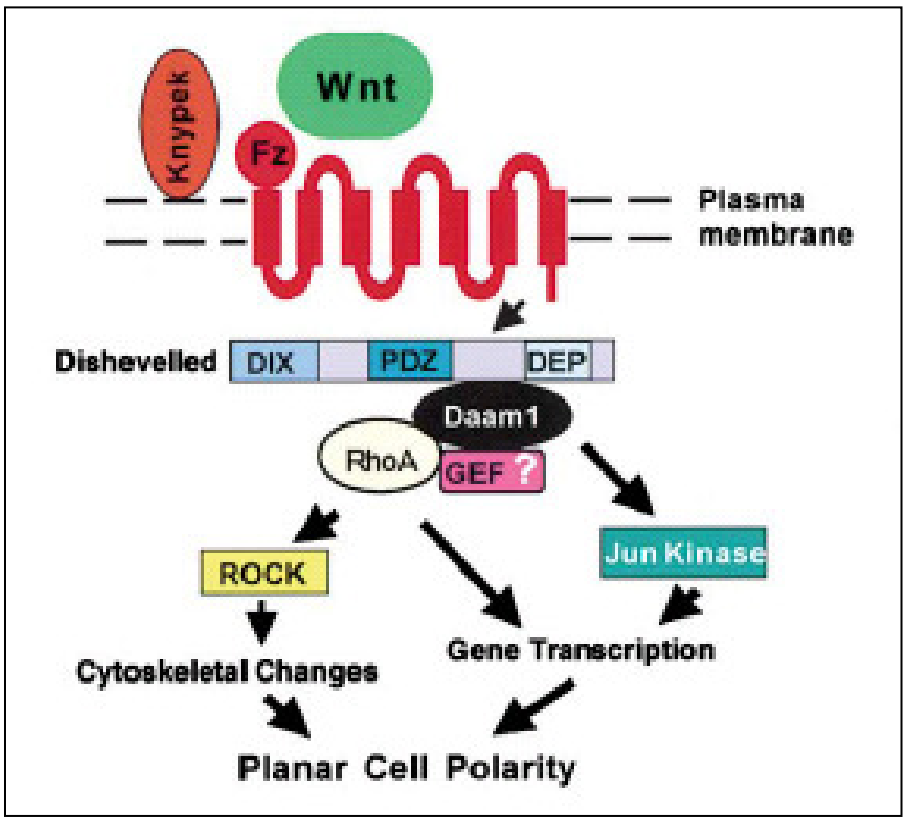


Figure I-8: The vertebrate equivalent of the Drosophila planar cell polarity pathway, which regulates convergence and extension movements (Photographs from Habas et al., 2001). A Wnt signal activates a Frizzled receptor, which recruits Dsh to the plasma membrane and promotes Dsh-Daam1-RhoA complex formation and RhoA activation, likely via the recruitment of a Rho-GEF by the Daam1 scaffolding protein. Activated RhoA generates polarized cytoskeleton remodeling via the ROCK kinase and perhaps also induces changes in gene expression. The zebrafish *knypek* gene product, a glypican, also participates in this pathway and facilitates Wnt signal reception. In addition, DEP domain of Dishevelled is required and sufficient for JNK (Jun N-terminal kinase) activation and affects planar cell polarity.

intracellular mediators like RhoA GTPase, Jun kinase or intracellular calcium concentration (Fig. I-8). In vertebrates, the functions of the ligands like Wnt 5, Wnt11, and wnt4, can be classified into the PCP pathway. Overexpression or inhibition of Wnt 5/11 expression can disrupt convergence and extension. The inhibition of Wnt5/11 expression was revealed by the shorter border axes of zebrafish mutants *silberblick* (*sib*; wnt11) and pipetail (ppt; wnt5) (Heisenberg et al., 2000; Kilian et al., 2003). This indicates that balanced Wnt signalling is important for normal gastrulation movements (Heisenberg et al., 2000; Moon et al., 1993; Rauch et al., 1997). The affect of the PCP pathway on cell morphology can help explain why cell migration is influenced. PCP signalling can regulate both the length-to-width ratio of cells and their orientation with respect to the embryonic axes. In the *trilobite* (Van Gogh-like 2/Strabismus) mutant, both the length-to-width ratio and mediolateral alignment of paraxial ectodermal cells are reduced (Jessen et al., 2002), and these mutant cells move with reduced net dorsal speed along less-direct trajectories compared to wild-type cells. PCP signalling also controls cell division orientation which was proposed as being correlated with dorsal extension (Gong et al., 2004). Epiblast cells in dorsal tissues preferentially divide along the animal-vegetal axis of the embryo. Inhibiting the establishment of this animal-vegetal polarity by blocking wnt11, Dishevelled, or Strabismus (trilobite) disrupts this orientation and thus reduces the extension of the axis.

Receptors for PCP Wnt signalling, the frizzled receptors, including Frizzled 2 and 7 receptors, function in transferring the signal. Loss of function of Frizzled 2 and 7 causes defects in axis elongation and convergence extension (Matsui et al., 2005; Sumanas and Ekker, 2001). Another membrane protein, *knypek* (*kny*), which encodes the heparin sulfate proteoglycan, also participates in this movement. The *kny* mutant embryo shows anteroposterior

shortening and mediolateral expansion of the axis (Topczewski et al., 2001). *kny* can enhance abnormalities of prechordal plate migration. However, the molecular mechanism of noncanonical Wnts for either of the Frizzled receptors is still unknown, and only Wnt5 is known to bind to ROR2 (a receptor tyrosine kinase) and may form a complex with Frizzle 2/5 receptors (Oishi et al., 2003). There is another transmembrane protein, strabismus (Stbm, also known as Van Gogh), which also controls CE movements and cell fates (Darken et al., 2002; Jessen et al., 2002; Park and Moon, 2002).

The central intracellular mediator for the PCP pathway is Dishevelled (Dsh) which mediates both the canonical and non-canonical Wnt pathways and separates these two signals (Boutros and Mlodzik, 1999). This specificity may come from different properties in different domains. The DIX domain is essential for β-catenin activity, whereas the PDZ and DEP domains are required for PCP signalling (Habas et al., 2001; Topczewski et al., 2001). Dsh interacting proteins, including Rho GTPase and Jun N-terminal kinase (JNK), can also control CE movement (Marlow et al., 2002; Oishi et al., 2003; Rui et al., 2007). In addition, JNK also contributes to the β-catenin pathway.

There is another pathway, the β-catenin-Stat3-Liv1-Snail1 pathway involved in CE movements especially on the prechordal plate migration (Miyagi et al., 2004; Yamashita et al., 2002; Yamashita et al., 2004). β-catenin activates an unknown ligand for the JAK/STAT pathway, cumulating in the phosphorylation and consequent activation of the transcription factor Stat3. The zinc transporter, Liv1, is activated by Stat3 and promotes the accumulation of the zinc finger transcription factor, snail, into the nucleus. This possibly promotes epithelial mesenchymal transformation (EMT) in anterior axial mesoderm cells. Blocking STAT3 function using morpholinos results in severe reduction of convergence and extension movement. As

phosphor-STAT3 accumulates specifically on the dorsal side in response to β-catenin stabilization, the signal regulated by Stat3 might provide polarity cues (Yamashita et al., 2002; Yamashita et al., 2004). However, further genetic and cell biology studies are required to more thoroughly test this model.

β-Catenin-regulated genes might also be an attractant signal for migration, as shown by the ventrally localized β-catenin injection which redirects cell migration ventrally (Schier and Talbot, 2005). This redirection of cell migration can also be seen in local FGF8 signal overexpression (Furthauer et al., 1997). Both of these molecules can eliminate BMP signalling expression, and combined results show that BMP signalling can repel cell migration (von der Hardt et al., 2007), which indicates that BMP signalling not only controls the patterning but also affects cell migration. However, the effect on cell migration by BMP signalling may be independent; in dorsalized swirl/bmp2b mutants, convergence is reduced but extension is normal or even increased (Myers et al., 2002a). In addition, the absence of the polysaccharide, hyaluronan, blocks convergence but not extension (Bakkers et al., 2004). This indicates that convergence and extension may be linked but may also be independent.

There are many other molecules that are involved in gastrulation movements or cell polarity including phosphoinnositide 3-kinase in the PDGF/PI3K/PKB pathway (Montero et al., 2003), the G-protein, Gα12/13 (Lin et al., 2005), hyaluronan (Bakkers et al., 2004), cyclooxygenase-1 (Cha et al., 2005), estrogen receptor-related α (Bardet et al., 2005), Scribble 1 (Wada et al., 2005), Fyn/Yes (Jopling and Hertog, 2007), Nemo-like kinase (Thorpe and Moon, 2004), Ephrins (Chan et al., 2001; Oates et al., 1999), cyclase-associated protein-1 (Cap1) (Daggett et al., 2004), and slit (Yeo et al., 2001). Most of them interfere with CE movements without changing a cell's

fate. However, the actual mechanism for most molecules still needs to be established.

2. Proteins that are linked to cell fate determination: the concept of BMP, Nodal, and Wnt signalling

There are four common signalling pathways that contribute to early zebrafish development: BMP (bone morphogenetic protein), Wnt, FGF (fibroblast growth factor), and Nodal pathways (the TGFβ family). The expression and proposed activity region in early zebrafish embryos (in the shield stage) are shown in Fig. I-9. These signals can all contribute to controlling dorsal-ventral and anterior-posterior asymmetry.

In the wild-type situation, the fate map is as shown in Fig. I-4 as described above. When the embryo loses control of the signals that control early patterning, it can become anteriorized, posteriorized, ventralized, or dorsalized.

(1) Dorsalization

When an embryo overexpresses a dorsalized signal like Chordin, which can bind to the ventralized signal, BMP, and inhibit its function, the embryo becomes dorsalized (Fig I-10B). The entire ectoderm is covered by the neuroectoderm, and the dorsal mesoderm region, like notochord and somite, has extended toward the ventral side of the embryo. The phenotype of the embryo will consist of an intact head, a curved trunk containing only the notochord and a few somites.

(2) Ventralization

When an embryo overexpresses a ventralized signal like BMP, the ventral ectoderm enlarges to cover the entire dorsal ectoderm (Fig I-10C). The ventral

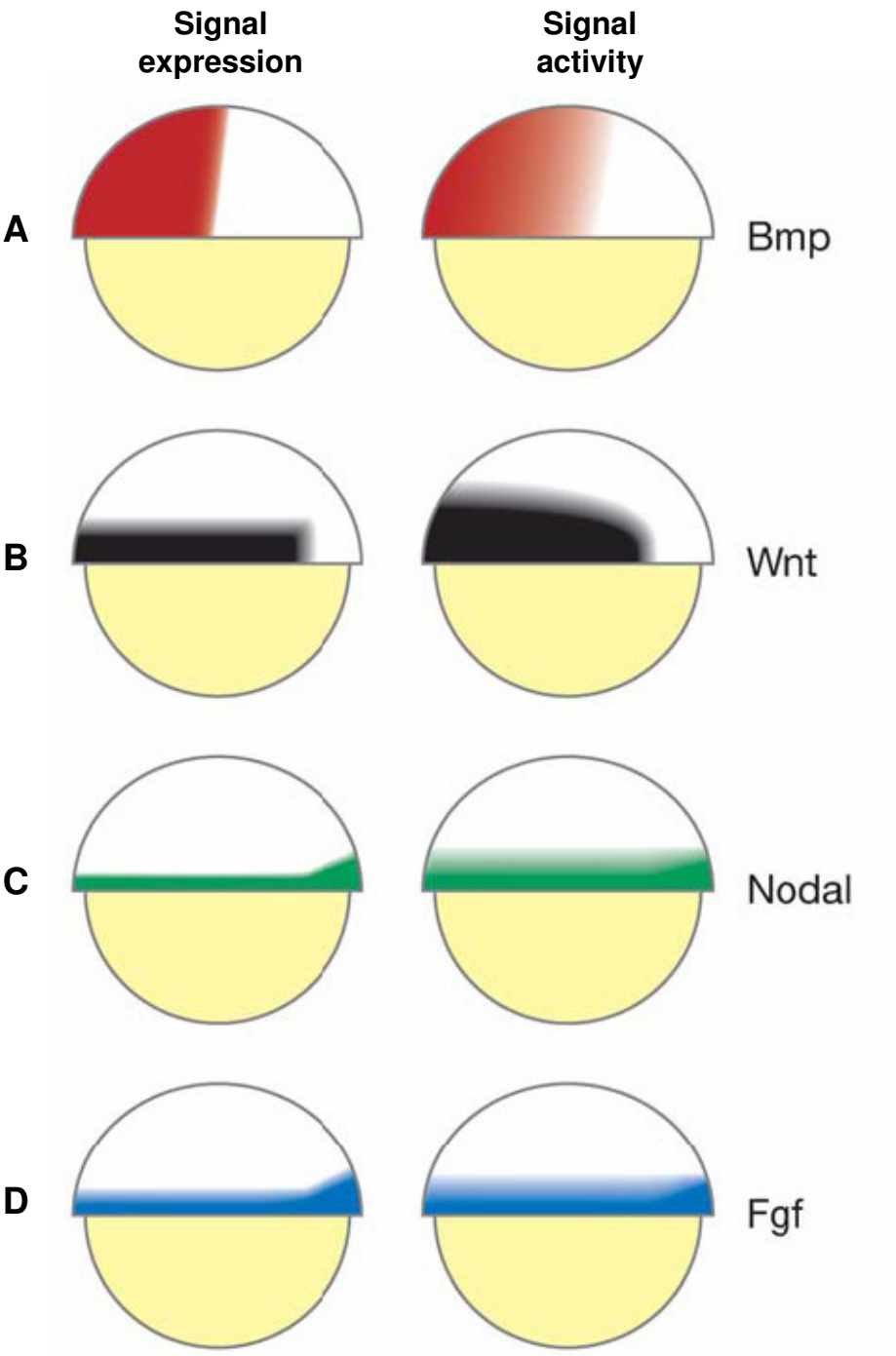


Figure I-9 : Signals patterning the embryo. The picture is from Schier and Talbot 2005. All the embryos are at late-blastula stage, lateral view, dorsal to the right, animal pole to the top. Signal expression is based on published reports, but signaling activities are speculative and based on the potential range of signals and the expression pattern and range of antagonists. Nodal and FGF signals are concentrated on the dorsal side soon after the mid-blastula transition (not shown), but these signals are more uniform across the dorsal-ventral axis by the late-blastula stage that is represented in the figure.

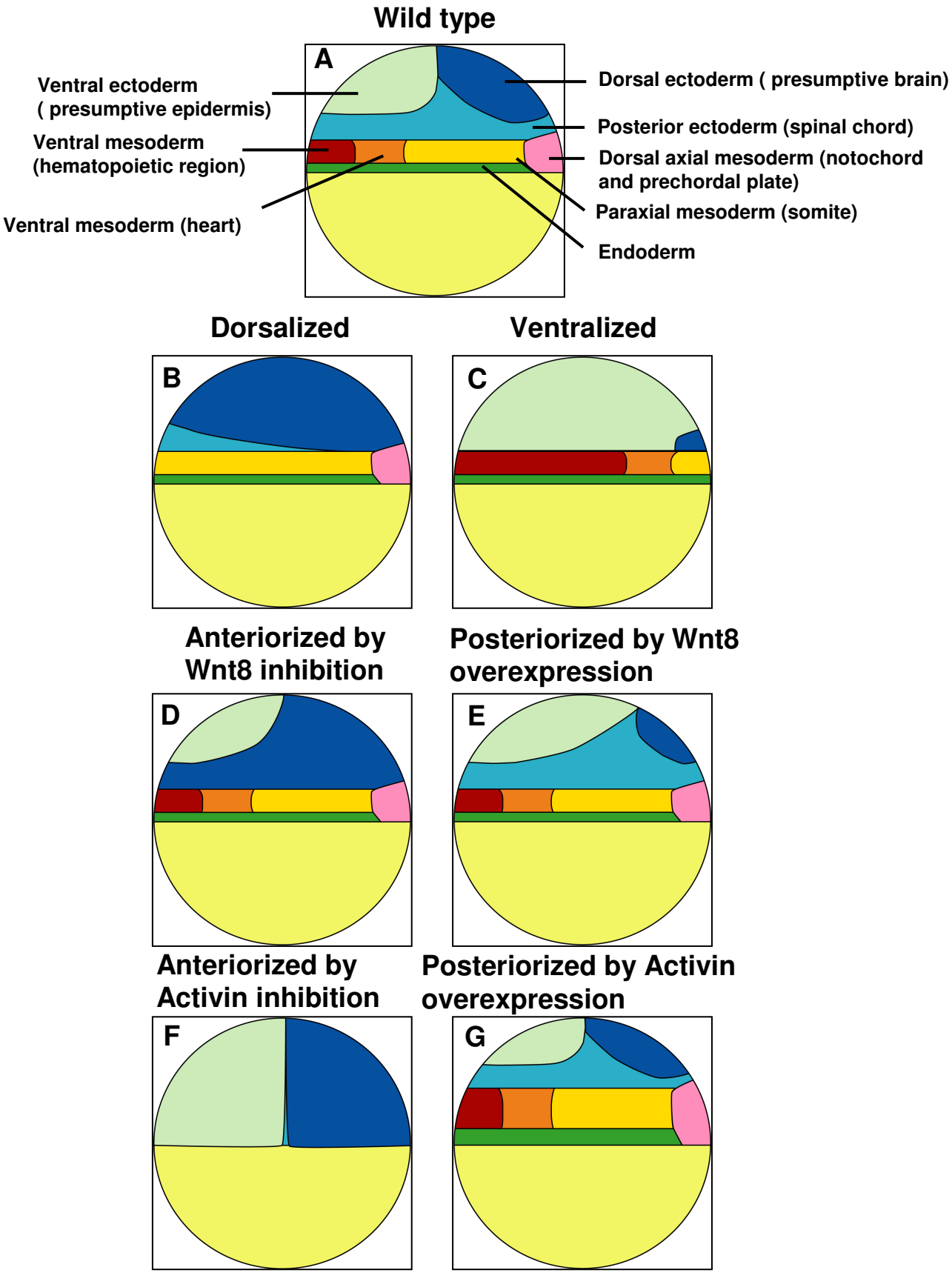


Figure I-10 : Example of phenotypes affecting early development. The phenotypes are illustrated using the fate map. (A). The phenotypes include dorsalized (B), ventralized (C), anteriorized by Wnt8 inhibition (D) or Activin inhibition (F), posteriorized by Wnt8 overexpression (E) or Activin overexpression (G). All the embryos are lateral view and anterior (animal pole) to the top, posterior (Vegetal pole) to the bottom, dorsal to the right and ventral to the left.

mesodermal region is also enlarged toward the dorsal side. The resulting embryo only has hematopoietic territory, somites, epidermic tissue and no cephalic region.

(3) Anteriorization

When an embryo becomes anteriorized due to zygotic Wnt 8 signalling inhibition (Fig I-10D) or Activin inhibition by overexpressed the Activin antagonist, Antivin (Fig I-10F), the presumptive brain, covers the dorsal and ventral posterior parts of the ectoderm in zygotic Wnt8 knock down embryos or covers the dorsal margin most regions including the mesendoderm layer in Antivin overexpression embryos (Thisse et al., 2000; Thisse and Thisse, 1999), respectively. This increase in the brain region in the ventral posterior ectoderm due to zygotic Wnt8 knock down will not concern the ventral anterior ectoderm and some cells still maintain the ventral anterior ectodermal marker fate.

In Antivin overexpressed embryos, ventral anterior ectoderm even increases more dorsally and vegetally to the margin. The phenotype of anteriorized embryos shows a large head with relatively normal ectoderm but lost or reduction of the mesendoderm structures and a reduced tail.

(4) Posteriorization

When an embryo becomes posteriorized due to overexpressing zygotic Wnt8 signalling (Fig I-10E), the anterior neuroectoderm decreases and this territory is replaced by ventral anterior ectoderm. The posterior ectoderm and mesoderm are still intact. Posteriorized fish only lose the brain but still have an intact trunk and tail.

When an embryo becomes posteriorized due to up-regulating the Activin activity by inhibition of Antivin (Fig I-10G, (Agathon et al., 2001)), the posterior ectoderm cell fate is replaced by mesoderm and endoderm fate. Posteriorized

fish display hypertrophic axial mesoderm with a strong excess of hatching gland and notochord.

a. Presentation of the BMP pathway

BMPs (Bone Morphogenetic Protein) belong to the TGFβ (Transforming Growth Factor beta) family that also includes TGFβs and activins, except BMP1, which is a metalloprotease. BMPs were originally identified as molecules that can induce ectopic bone and cartilage formation in rodents (Wozney et al., 1988). BMPs are conserved across the animal kingdom including vertebrates, arthropods, and nematodes. In *Drosophila*, the BMP ligands, Decapentaplegic (Dpp), have been shown to regulate dorsal-ventral (DV) patterning (Raftery and Sutherland, 1999). In vertebrate, *Xenopus*, BMP2, 4, and 7 ventralize the early mesoderm and act as negative regulators of neuralization (Harland, 1994; Hemmati-Brivanlou and Melton, 1997). In zebrafish, BMP2b, 4, and 7 are also required to maintain the ventral cell fate including the lateral margin fates such as blood, heart, pronephrons, and tail somites and also the ventral ectoderm cell fate but not the dorsal cell fate (Little and Mullins, 2006).

Transduction of BMP signals involves two distinct types of transmembrane serine/threonine kinase receptors, types I and II (Fig. I-11). When BMPs bind to a receptor, the closely related Smad family transcription factors, Smad1/5/8, are phosphorylated by the receptors, allowing these proteins to be translocated to the nucleus and regulate target gene expression together with the nonreceptor-regulated Smad protein, Smad4, and other DNA-binding cofactors. In zebrafish embryos, the maternal BMP signal is necessary to activate zygotic BMP expression. This conclusion was drawn from female smad5 homozygous mutants which can produce stronger dorsalized progeny

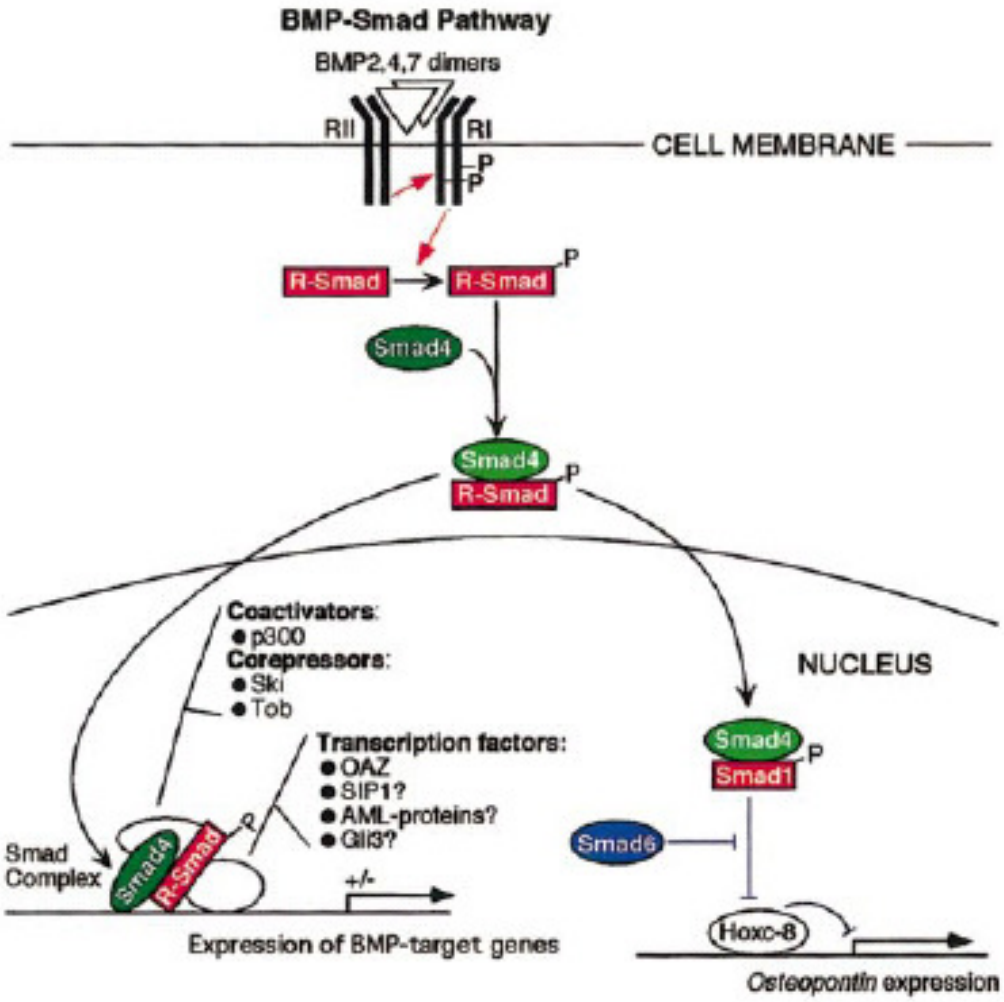


Figure I-11:The BMP–Smad pathway (Photograph from von Bubnoff and Cho, 2001). BMP2, -4, or -7 dimers bind to the receptor complex, leading to phosphorylation of the type I receptor (RI) by the type II receptor (RII), which in turn phosphorylates an appropriate R-Smad (Smad1, -5, or -8). This phosphorylation enables the R-Smad to complex with the Co-Smad, Smad4, and the R-Smad/Smad4 complex enters the nucleus to activate or repress target genes depending on which nuclearcofactors are present Red indicates activation and blue indicates inhibition. Smads that are part of or regulate the BMP-Smad pathway are colored: Smad1, -5, and -8 are red, the Co-Smad Smad4 is green, and the Smad6 are blue.

than mutants from heterozygous parents (Kramer et al., 2002). Other mutants include different BMPs (Dick et al., 2000; Kishimoto et al., 1997; Nguyen et al., 1998; Schmid et al., 2000), the type I receptor Alk8 (Bauer et al., 2001; Mintzer et al., 2001), the transcriptional effector, Smad5 (Hild et al., 1999), and the protease, Tolloid (Connors et al., 1999), which cleave the Bmp antagonist, Chordin, all of which show different degrees of dorsalization. In addition, there are also intracellular modulators that can regulate the BMP activity gradient. There are inhibitory Smads, Smad6/7, in the intracellular matrix which interfere with Smad 1/5/8 phosphorylation (Fig I-11). In addition, Bambi (BMP and Activin membrane bound inhibitor) which is induced by BMP2/4 is a transmembrane protein that functions as a decoy receptor. Bambi shows sequence similarity to TGF-β receptors but lacks intracellular kinase domain and can reduce the BMP signalling through formation of non-functional complex with other TGF-β receptors when binds BMPs (von Bubnoff and Cho, 2001).

Finally there are extracellular BMP activity-regulatory proteins. Chordin, Noggin, Follistatin, Ogon/Sizzled, Tolloid, BMP1a, ADMP (antidorsalizing morphogenetic protein), and Twisted gastrulation can produce a regulatory network to regulate BMP activity (Yamamoto and Oelgeschlager, 2004). Chordin, Noggin, and Follistatin can bind to BMP molecules and prevent them from binding to receptors (Yamamoto and Oelgeschlager, 2004) and inhibit BMP signalling. Sizzled is a secreted Frizzled-related protein (SFRP) but functions as a competitor for Chordin with Bmp1a in zebrafish or Xolloid-related (Xlr) in *Xenopus,* both of which are metalloproteinases and can degrade Chordin (Lee et al., 2006; Muraoka et al., 2006). Thus the role of Sizzled is to prevent Chordin degradation, and it ultimately inhibits BMP signalling (Muraoka et al., 2006). BMP1a and *tolloid* (*tll1*) have been

intensively studied in zebrafish, and both are metalloproteases and function to cleave Chordin (Connors et al., 1999). The overexpression of Tll1 produces phenocopies of the *chd* mutant (Connors et al., 2006). In contrast, the phenotype of a tll1 (*mini fin*) and Chordin double mutants is also equivalent to the *Chd* mutant. This indicates the specificity of Tll1 for Chordin cleavage (Wagner and Mullins, 2002). The function of Twisted gatstrulation (Tsg) is still not clear, because loss of function by Tsg produces both dorsalized (Little and Mullins, 2004; Xie and Fisher, 2005) and ventralized phenotypes (Ross et al., 2001). The results of dorsalization due to loss of Tsg function shows that this dorsalization can partially rescue the ventralized phenotypes of *chordin* and *ogon/sizzled* mutants. This indicates the Tsg must have functions independent of Chordin and its fragments. The ADMP has BMP-like activity, can bind to both Chordin and Tsg, and signals via the ALK-2 receptor. The ADMP can serve in a self-regulatory role on the dorsal part of the embryo and will increase the expression after ventral BMP2/4/7 signals are deplete (Reversade and De Robertis, 2005).

b. The FGF pathway

Fibroblast growth factor (FGF) signalling occurs through binding of FGFs to FGF receptors (FGFRs), and then the signal is transmitted by tyrosine phosphorylation. FGFs in zebrafish consist of 10 isoforms, including FGF2~4, 6, 8, 10, 17a, 17b, 18, and 24. FGFRs exist as inactivate monomers, which are activated when two FGF molecules through heparin sulfate proteoglycan bind to the extracellular IgII and IgIII domains of a receptor leading to its homodimerization (Fig. I-12; (Schlessinger et al., 2000)). The main signalling activated through the stimulation of FGFRs is the RAS/MAP kinase pathway. Through sequential intermediate activation, the MAP kinase (ERK1,2)

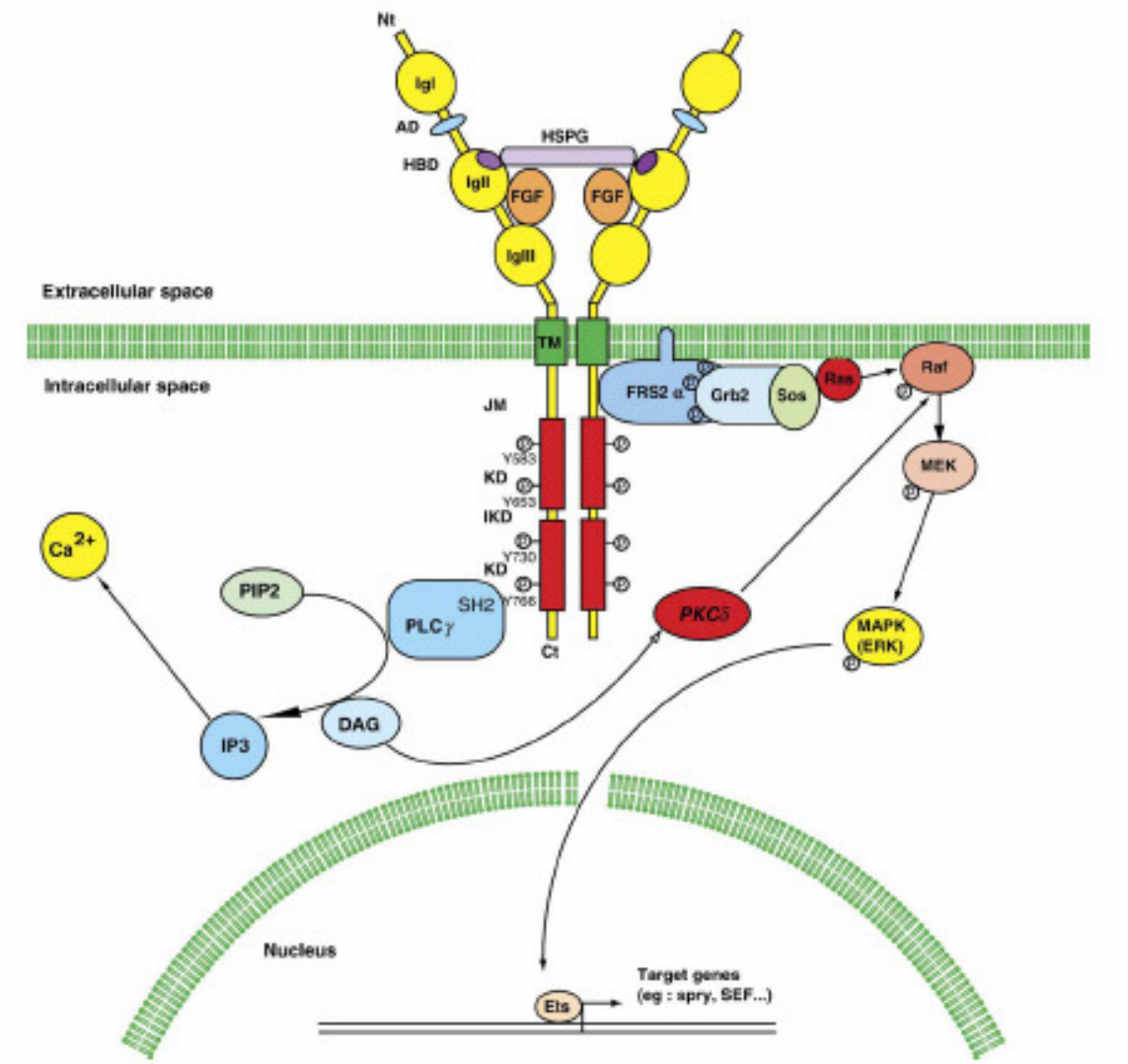


Figure I-12. : FGF receptors and FGF signal transduction (Photograph from Thisse, 2005). FGFRs are modular proteins comprising 3 immunoglobulin domains (IgI, IgII and IgIII). FGF ligands linked to heparin sulfate proteoglycan (HSPG) bind to IgII and IgIII of FGFR. This results in the dimerization and the subsequent transactivation by phosphorylation of specific tyrosine residues. The main two transduction pathways involve the phospholipase C-g (PLCg) and the Ras/MAP kinase. The SH2 domain of the PLCg interacts with the phosphorylated Y766 of the activated receptor. The activated PLCg hydrolyzes the phosphatidyl-inositol-4,5-diphosphate (PIP2) to inositol-1,4,5-triphophate (IP3) and the diacylglycerol (DAG). IP3 releases Ca^{2+} while DAG activates the protein kinase C-y (PKCy). Activated PKCy activates Raf by phosphorylating its S338 and stimulates the downstream pathway in a Ras independent manner. The another main pathway involves the interaction of the docking protein FRS2a with the amino-acid residues 407– 433 in the FGFRs (Xu et al., 1998). This protein is activated by phosphorylation on multiple tyrosine residues and subsequently interacts and activates Grb2 linked to Sos, a nucleotide exchange factor involved in the activation of Ras. Activated Ras then activates Raf which stimulates MEK which in turn phosphorylates the MAP kinase ERK. This last activated component translocates to the nucleus and phosphorylates specific transcription factors of the Ets family which in turn activate expression of specific FGF target genes. P: phosphorylation.

ultimately enters the nucleus and phosphorylates target transcriptors which contain the Ets domain (Sternberg and Alberola-Ila, 1998; Wasylyk et al., 1998).

Data from FGF signalling in zebrafish mainly suggest an early role of repressing BMP signalling and a later role of promoting the development of posterior structures. The expression of BMP genes, initially activated in the entire blastula, becomes progressively restricted ventrally. This ventral restriction coincides with the spread of FGF activity from the dorsal side of the embryo, implying that FGF signalling in the dorsal portion downregulates BMP gene expression. Consistently, general activation of the FGF/Ras/MAP kinase signalling pathway inhibits BMP gene expression in the entire blastula. Conversely, inhibition of FGF signalling causes BMP gene expression to increase dorsally, leading to expansion of ventral cell fates. Altogether, this shows that FGF signalling is essential for delimiting the expression domain of BMPs in blastula-stage embryos. Therefore, FGFs act upstream of ventral morphogens and function as initial signals for establishing dorsal-ventral patterning. However, blocking only the FGF signalling does not result in a ventralized embryo, but the combination of FGF and Chordin loss of function results in a ventralized embryo (Furthauer et al., 2004). This suggests that the FGF, Bozozok/Dharma, and BMP antagonists such as Chordin and Noggin all contribute to blocking BMP signalling in dorsal margin blastomeres.

The range of FGF signalling can encompass a long distance from the vegetal to the animal pole. This signalling distance is dependent on receptor-mediated endocytosis, which leads to clearance of the ligand from the extracellular space. Subsequently, blocking endocytosis results in an increased FGF signalling range (Scholpp and Brand, 2004). This long-range signalling result is consistent with the expression of FGF downstream genes

like pea3, erm, and sprouty4, which are induced in broad domains in neighboring cells (Furthauer et al., 2004; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001; Tsang et al., 2004).

Evidence that FGFs are involved in the priming and maintenance of the embryo posterior development is from FGF signalling loss of function. Severe posterior truncations are generated in embryos exposed to the FGFR inhibitor, SU5402, a dominant negative FGF receptor lacking full *fgf8* and *fgf24* activity (Draper et al., 2003; Griffin et al., 1995; Griffin and Kimelman, 2003; Mathieu et al., 2004). The tail and posterior trunk mesoderm do not form in these embryos, and mesodermal markers such as the T-box genes, *ntl* and *spt*, are lost at the gastrula stage. These data show that FGF signalling is required for a pool of mesoderm progenitors during gastrulation.

FGF signalling also contributes to mesoderm formation, but this only begins at a later gastrulation stage. This conclusion comes from the expression of mesodermal markers like tbx6, spt, and ntl, which are lost at the later gastrula stage but are intact in the early stage in FGF loss of function (Draper et al., 2003; Furthauer et al., 2002; Furthauer et al., 2004; Griffin et al., 1995; Griffin and Kimelman, 2003; Mathieu et al., 2004; Raible and Brand, 2001; Tsang et al., 2002; Tsang et al., 2004). There is also evidence which shows that FGFs have a redundant role in Nodal signalling during mesoderm formation. This is from the result that partial FGF signalling inhibition and the Nodal signalling mutant, one-eyed pinhead (Nodal co-receptor), disrupts posterior development and causes dorsal mesoderm cell death by the end of gastrulation (Griffin and Kimelman, 2003; Mathieu et al., 2004). However, the real mechanism is still unknown.

FGF signalling can be regulated by various molecules and becomes an inhibitory or activation feedback loop (Thisse and Thisse, 2005). These signals

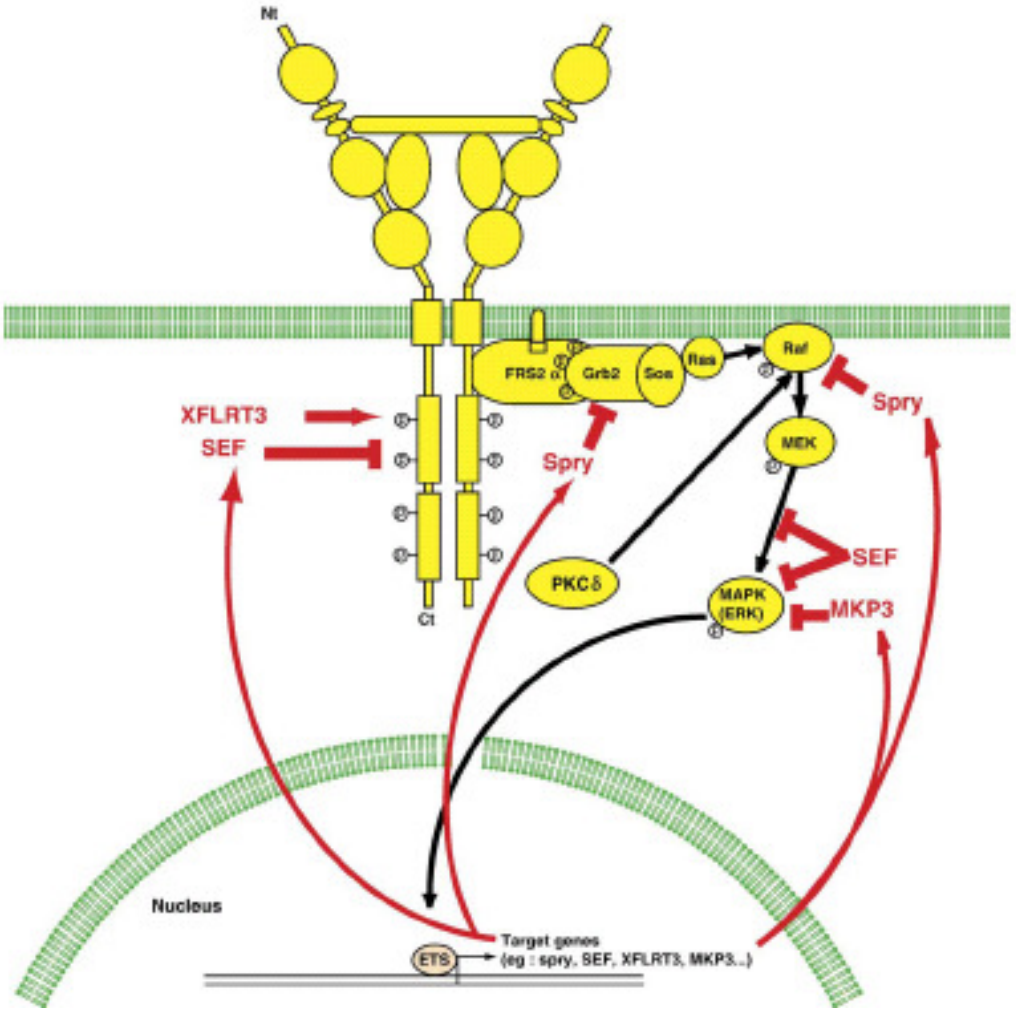


Figure I-13 : FGF signaling and its regulation (Photograph from Thisse, 2005). The stimulation of FGFR by FGF ligands results in the activation of specific target genes. Among them are several feedback inhibitors which are involved in the attenuation of FGF signaling. Spry acts at the level of Grb2 and/or at the level of Raf. Sef and XFLRT3 are both located at the membrane and interact with FGFR. Sef functions as a negative regulator while XFLRT3 enhances the FGF activity resulting in the phosphorylation of the MAP kinase ERK. Sef was shown also to affect the phosphorylation of the MAP kinase, either directly at the level of ERK or by preventing the phosphorylation of ERK by MEK. Finally, MKP3 negatively regulates the FGF signaling pathway by dephosphorylation of activated MAP kinase.

include Sprouty (Spry), XFLRT3, SEF, and MAP kinase phosphatase 3 (MAKP3). Spry functions on several intermediate molecules in the FGF signal pathway. Tyrosine-phosphorylated Spry can bind to Grb2 and interrupt the FGFR/Ras/Raf/MAPK pathway. Spry can also bind to Raf and inhibit Raf phosphorylation by PLCγ-activated PKCδ (Fig. I-13). XFLRT3, SEF, and MAKP3 were found by large-scale in situ hybridization screening and were isolated by comparison with the expression patterns of FGFs (Gawantka et al., 1998; Kudoh et al., 2001; Thisse et al., 2001b). The gain or loss of function of XFLRT3 in *Xenopus* was shown to phenocopy FGF signalling (Bottcher et al., 2004). But in zebrafish, this phenocopy cannot be seen. This suggests that FLRT3 might not be related to the FGF pathway (Thisse and Thisse, 2005). Sef (similar expression to FGF genes) functions as an antagonist to FGF signalling according to gain- and loss-of-function studies. Sef interferes with FGF signalling by acting downstream or at the level of MEK and upstream (or at the level) of MAP kinase (Furthauer et al., 2002). MAPK3 can negatively regulate the MAP kinase cascade through dephosphorylation of activated MAPK proteins. Upon binding of phosphorylated MAPKs to the MAPK-binding domain in MKP3, conformational activation of the C-terminal phosphatase domain is achieved, leading to the inactivation of MAPKs (Camps et al., 2000; Fjeld et al., 2000; Zhao and Zhang, 2001).

FGF signalling also functions on cell movement and acts as a chemoattractant for migrating cells. For example, FGF2 and FGF8 are potent chemoattractants for mesencephalic neural crest cell migration (Kubota and Ito, 2000), and FGF2 and FGF4 attract mesenchymal cells during limb bud development in the mouse (Li and Muneoka, 1999; Webb et al., 1997). FGF10 also exerts a potent chemoattractive effect in directing lung distal epithelial buds to their destination (Park et al., 1998). The function of FGF signalling can

also be used as a chemotactic for cell migration. For example, when forming the chick notochord, anterior streak cells can be chemorepulsed by FGF8 signalling and chemoattracted by FGF4 signalling (Yang et al., 2002).

c. The Nodal pathway

Nodal signalling molecules belong to the TGFβ superfamily and are essential inducers of the mesoderm and endoderm in vertebrates (Schier, 2003). Nodal signals bind to EGF-CFC co-receptors and type I and II Activin receptors which function as serine/threonine kinases. Then the receptor phosphorylates the transcription factors, Smad2 and Smad3. This results in the binding of Smad4 and its translocation into the nucleus where it associates with additional transcription factors, such as FoxH1 and Mixer, to regulate target genes (Fig. I-14). Nodal signalling is antagonized by feedback inhibitors such as Lefty proteins, which are divergent members of the TGFβ family and block EGF-CFC co-receptors (Chen and Shen, 2004; Cheng et al., 2004) and Antivin, which competitively binds to the type II Activin receptor (Thisse and Thisse, 1999) and Dapper 2, which enhances the degradation of type I Activin receptors (Zhang et al., 2004). The evidence showing the involvement of Nodal signalling in mesoderm and endoderm induction comes from loss- and gain-of-function studies. The absence of Nodal signalling in the *cyc;sqt* (two Nodal molecules) double mutant or the maternal-zygotic *one-eyed pinhead* (EGF-CFC co-receptor) mutant results in embryos that lack any endoderm or mesoderm, with the exception of a few somites in the tail (Feldman et al., 1998; Gritsman et al., 1999). In contrast, increasing Nodal signalling by loss of the antagonists, Lefty1 and Lefty2, or overexpression of Cyc or Sqt results in the fate transformation of ectodermal cells into mesoderm or ectoderm (Agathon et al., 2001; Chen and Schier, 2001; Chen and Schier, 2002; Erter et al., 1998;

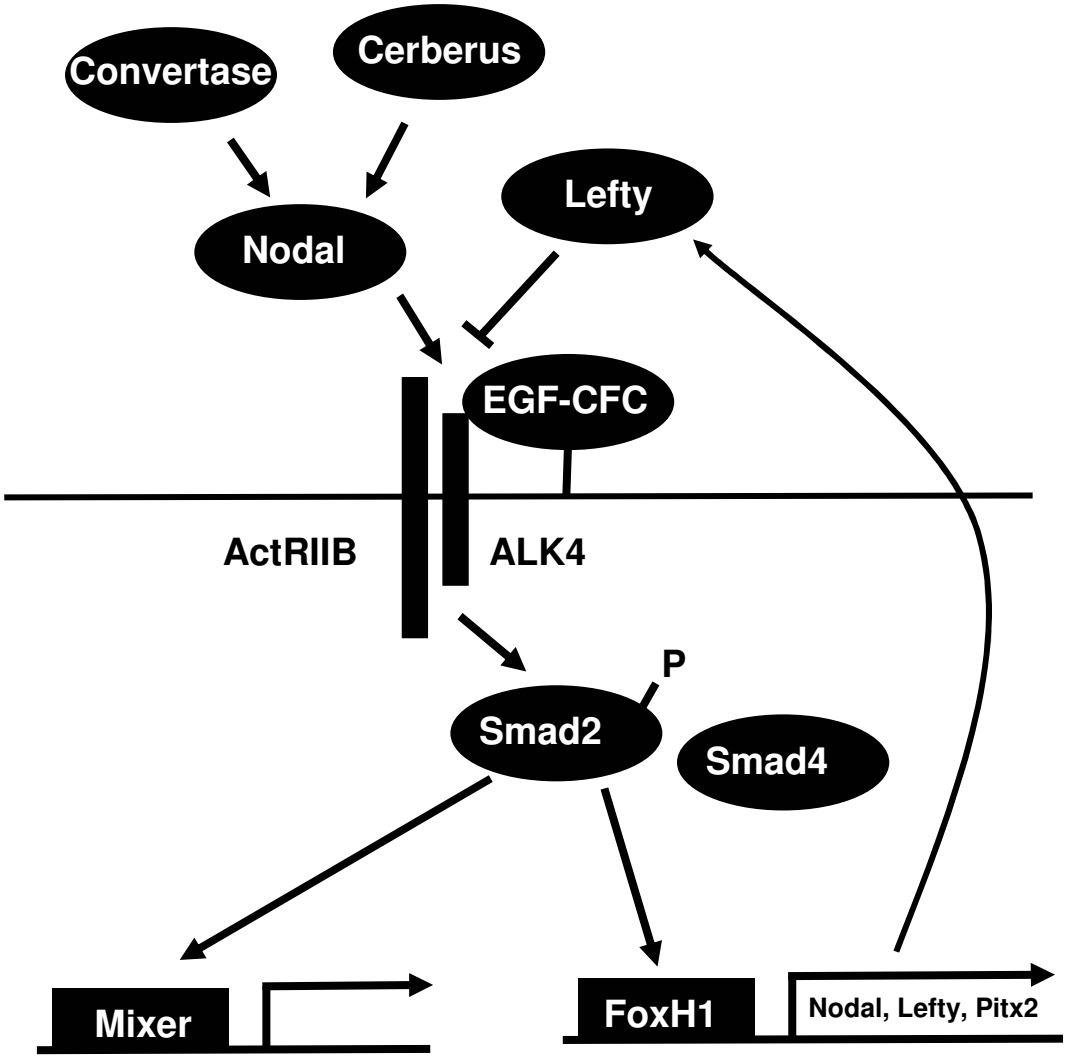


Figure I-14 : The Nodal signaling pathway (Photograph by Schier, 2003). Nodal signaling is activated by the interaction of Nodals with activin receptors (ActRIIB, ALK4) and EGF-CFC coreceptors, and it is inhibited by Leftys and Cerberus. Convertases process Nodal proproteins. Nodal signaling is transmitted intracellularly by phosphorylation of Smad2 and its association with Smad4 and transcription factors (FoxH1, Mixer) that determine which genes are regulated by P-Smad2. The induced transcription of Lefty by nodal signaling can form a feed back loop.

Feldman et al., 2002; Feldman et al., 1998; Gritsman et al., 2000; Rebagliati et al., 1998; Sampath et al., 1998). The same tendency was found in an experiment using *antivin* and *activin* as competitors for type II Activin receptors (Thisse et al., 2000); this shows that increasing *antivin* progressively deletes posterior fates within the ectoderm and mesendoderm, eventually resulting in the removal of all fates except the forebrain and eyes which are from anterior neuroectoderm cells. In contrast, overexpression of Activin or nodal-related factors converts the ectoderm that was fated to be forebrain into more-posterior ectodermal or mesodermal fates. It was proposed that modulation of intercellular signalling by Antivin/Activin and Nodal-related factors provides a mechanism for the graded establishment of cell fates along the entire embryo anteroposterior axis.

There is also a model for mesendoderm formation regulated by long- and short-range Nodal signalling in mutant and misexpression studies (Schier, 2003). The *sqt* and *cyc* genes are transcribed in cells closest to the margin, leading to local activity by both proteins. Sqt can move far away from the origin and induce mesendodermal markers in cells at distance (Chen and Schier, 2001). In contrast, Cyc only acts within a short range and induces mesodermal markers locally (Chen and Schier, 2001). Nodal signalling can be regulated by the antagonists, Lefty1 and Lefty2, which are also induced by Nodal and can become a feedback loop. The restriction can be locally inhibited by the expression of *sqt* and at a distance, can restrict the response to Sqt (Chen and Schier, 2002; Feldman et al., 2002).

d. The Wnt pathway

The Wnt signalling pathway described here is called the “canonical Wnt pathway” compared to the noncanonical Wnt pathway which functions mainly

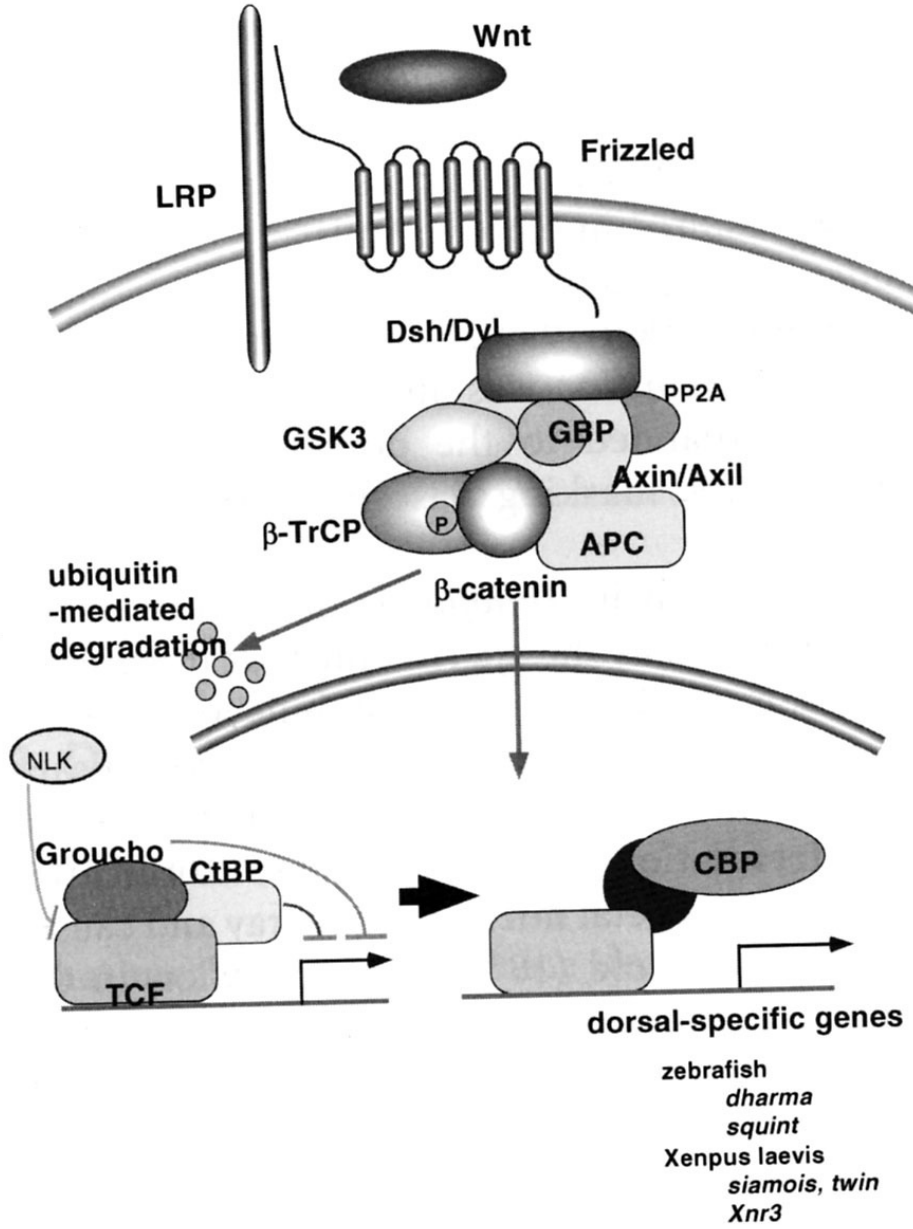


Figure I-15 : The canonical Wnt signaling pathway (Photograph from Hibi et al., 2002). In the absence of Wnt ligands, the serine/threonine kinase GSK3 and β-catenin form a complex with the scaffolding protein Axin and the tumor suppressor gene product APC, leading to phosphorylation of the amino-terminal region of β-catenin . Phosphorylated β-catenin is recognized by the F-box-motif-containing protein β-TrCP, a component of ubiquitin ligase, and is subjected to ubiquitination-dependent degradation. Upon stimulation by Wnt, Dsh suppresses the phosphorylation of β-catenin by GSK3 by an unknown mechanism, leading to the release and stabilization of β-catenin, which then moves to the nucleus and form a complex with Tcf or Lef factors. In the absence of β-catenin, high-mobility group (HMG) proteins in the Tcf/Lef family interact with the transcriptional corepressors, Groucho and CtBP, and/or with the histone deacetylase, HDAC1, and represses transcription of target genes, while the complex between Tcf/Lef and β-catenin activates transcription, possibly through interacting with the co-activator, CBP.

in cell movements and not in cell patterning. The canonical Wnt pathway begins when Wnt proteins bind to the Frizzled receptor family of seven transmembrane proteins to activate the downstream target, Dishevelled (Dsh/Dvl), as the next known component (Hibi et al., 2002) (Fig. I-15).

In the absence of Wnt ligands, the serine/threonine kinase, GSK3, and β-catenin from a complex with the scaffolding protein, Axin, and the tumor suppressor gene product, APC, leading to phosphorylation of the amino-terminal region of β-catenin. Phosphorylated β-catenin is recognized by the F-box-motif-containing protein, β-TrCP, a component of ubiquitin ligase, and is subjected to ubiquitination-dependent degradation.

Upon stimulation by Wnt, Dsh suppresses the phosphorylation of β-catenin by GSK3, leading to the release of stabilized β-catenin, which then moves into the nucleus and forms a complex with the amino terminal portion of the Tcf/Lef transcription factor. In the absence of β-catenin, high-mobility group (HMG) proteins in the Tcf/Lef family interact with the transcriptional corepressors, Groucho and CtBP, and/or with the histone deacetylase, HDAC1, and represses transcription of target genes (Billin et al., 2000; Brannon et al., 1999; Cavallo et al., 1998; Roose et al., 1998), while the complex between Tcf/Lef and β-catenin activates transcription, possibly through interacting with the co-activator, CBP (Takemaru and Moon, 2000).

The secreted molecules that can regulate Wnt signalling have been quite extensively studied, and they are all classified in the secreted Frizzled-related protein (sFRP) family. I introduce these molecules in the introduction to part III.

The Wnt signalling intermediate, β-catenin, can be maternally transferred into the oocyte and specifically accumulates in nuclei of dorsal margin blastomeres as early as the 128-cell stage (Schneider et al., 1996). The result of inhibiting this maternal β-catenin by the mutants, *ichabod* and *tokkaebi*

(through disruption of the stabilized form of β-catenin), shows that the embryo becomes ventralized and indicates that maternal β-catenin is necessary for dorsal axis formation (Kelly et al., 2000; Nojima et al., 2004). The same result can also be seen with RNA (cadherin or a dominant negative form of Tcf3 that binds to β-catenin but not DNA)-injected embryos (Pelegri and Maischein, 1998). The maternal β-catenin can induce several zygotic genes including *bozozok* (*boz*, also known as *dharma* and *nieuwkoid*), *chordin*, *dickkopf1* (*dkk1*), and *squint* (*sqt*), and FGF signals (Dorsky et al., 2002; Dougan et al., 2003; Fekany et al., 1999; Feldman et al., 1998; Furthauer et al., 2004; Hashimoto et al., 2000; Kelly et al., 2000; Koos and Ho, 1998; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001; Ryu et al., 2001; Shinya et al., 2000; Tsang et al., 2004; Yamanaka et al., 1998). These maternal β-catenin downstream targets act to inhibit the action of ventralizing factors or in the case of Sqt, induce mesendodermal fates at the dorsal margin. There is evidence showing that maternal Wnt 11 signalling is necessary for maternal β-catenin accumulation in dorsal blastomeres in *Xenopus* (Tao et al., 2005). However, this function was not found in zebrafish maternal and zygotic Wnt 11 mutants (*silberblick*) (Heisenberg et al., 2000), which only controls cell movement.

Wnt signalling most frequently studied in the zebrafish is Wnt8 and Wnt3a. Loss of function of Wnt8 showed that it is required for establishing ventral and posterior fates (Erter et al., 2001; Lekven et al., 2001). Wnt8 and Wnt3a have similar functions in tail formation and are redundant (Shimizu et al., 2005a). Loss of both Wnt 8 and Wnt 3a functions results in stronger expansion of dorsoanterior fates. In addition, influence on the anterior-posterior axis of the neural ectoderm by Wnt8 loss of function supports a role for Wnt 8 in posteriorizing the neuroectoderm (Erter et al., 2001; Lekven et al., 2001).

Research on Wnt8 downstream target genes found three ventral expression structure-related transcription repressors, Vox(Vega1)/ Vent(Vega2)/Ved, which can repress dorsal genes such as *chordin*, *bozozok* (*Dharma*), *goosecoid*, *floating head* (*flk*), and *dkk1* (Ramel and Lekven, 2004). In contrast, Dharma can repress the expression on *bmp2b* and *wnt8* (Fekany-Lee et al., 2000; Gonzalez et al., 2000; Imai et al., 2001; Leung et al., 2003). This suggests a model for dorsal ventral patterning by dorsal maternal β-catenin downstream zygotic genes against the ventral zygotic BMP and Wnt downstream genes (Schier and Talbot, 2005). In the beginning of zygotic gene expression, ventral *vox* (possibly activated by maternal BMP and zygotic Wnt8), overall *bmp2b* (possibly activated by maternal BMP), ventral *vent* (activated zygotic Wnt8), and dorsal β-catenin downstream genes including *Dharma* are co-expressed at the same time (Imai et al., 2001; Kramer et al., 2002; Ramel and Lekven, 2004; Sidi et al., 2003). Soon after, Dharma represses transcription of bmp2b, vox, and other ventralizing genes at the dorsal margin (Fekany et al., 1999; Kawahara et al., 2000a; Leung et al., 2003). This allows expression of dorsal genes, such as chordin, dkk1, and goosecoid, which would otherwise be repressed by vox/ved/vent (Imai et al., 2001; Shimizu et al., 2002). As a result, this becomes the first wave of zygotic gene expression characterized by dorsal cells (e.g., *dharma*, *goosecoid*, *chordin*, and *dkk1*) or ventral cells (e.g., *bmp2b*, *bmp7*, *vox*, *vent*, *ved*, and *wnt8*) (Fig I-16A). After this first wave of zygotic gene expression, the subsequent pattern becomes more elaborate. *vox*/*vent* and *bmp2b*/*swirl,* which are initially independent of each other's actions, become interdependent and may form a positive feedback loop with each other during gastrulation (Imai et al., 2001; Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000) (Fig I-16B). This positive feedback loop maintains ventral positional identity during

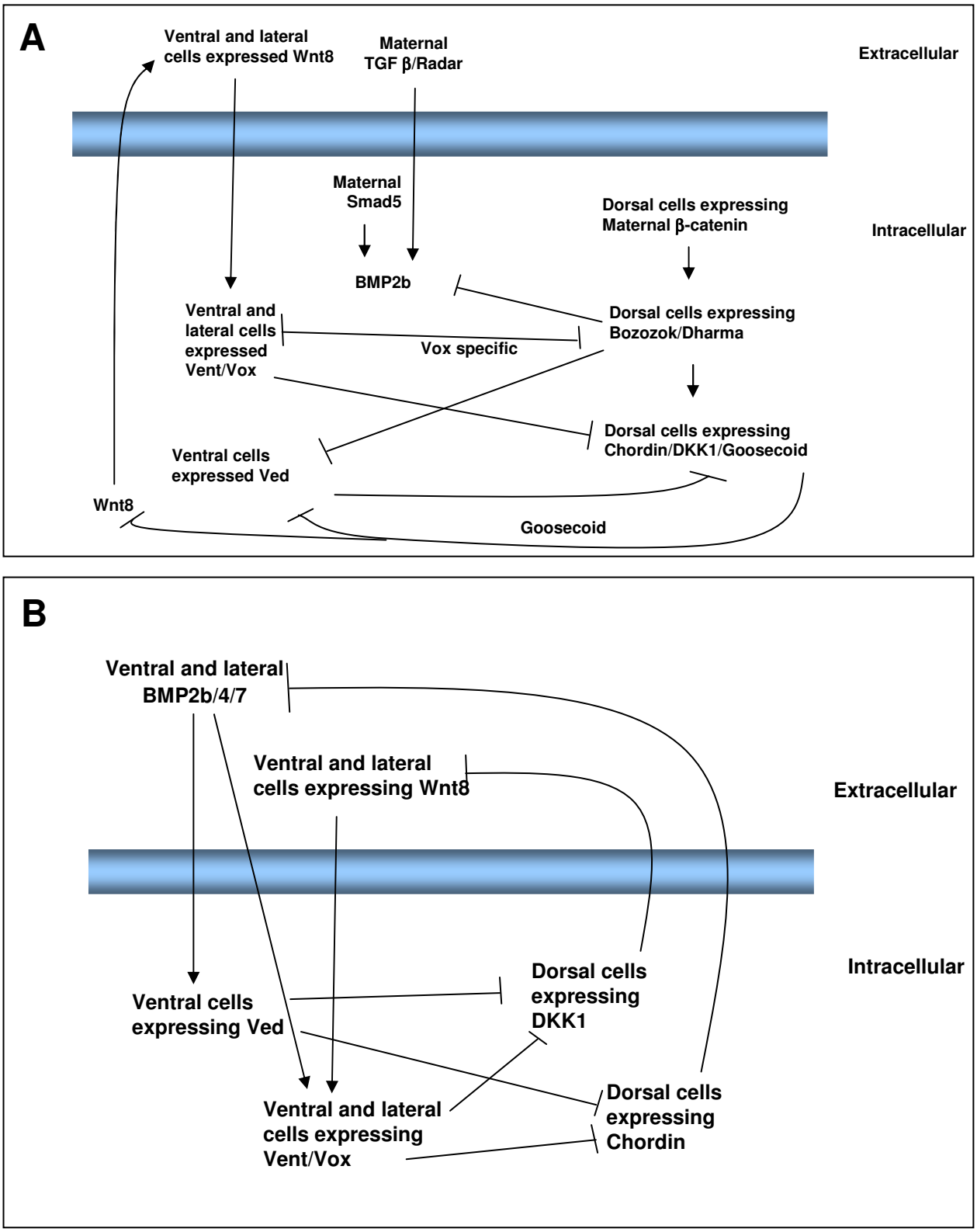


Figure I-16 : The interaction between ventral Wnt8 and BMP and dorsal maternal β-catenin signalling in the early zebrafish embryo. In the beginning of zygotic gene expression (A) Late Blastula (zygotic genes expression starts, 30% epiboly-shield), ventral ved/vent/vox, overall bmp2b and dorsal β-catenin downstream genes including Dharma are co-expressed at the same time. Soon after, Dharma represses transcription of bmp2b, vox, and other ventralizing genes at the dorsal margin. This allows expression of dorsal genes, such as chordin, dkk1, and goosecoid, which would otherwise be repressed by vox/ved/vent. During Mid-gastrula stage (B) (70 % epiboly), the subsequent pattern becomes more elaborate. ved/vent/vox and bmp2b, which are initially independent of each other's actions, become interdependent and may form a positive feedback loop with each other during gastrulation. This positive feedback loop is through inhibiting dorsal genes expression like DKK1 and Chordin then preventing their function on ventral extracellular BMP2b/4/7 or Wnt8 inhibition. Finally the ventral BMP2b/4/7 or Wnt8 can induce the ved/vent/vox expression.

gastrulation, and the participation of the extracellular factors, Chordin and BMP, incorporates flexibility and sensitivity into the cellular environment through the mechanism that maintains dorsal-ventral identity. This feedback loop allows cells to migrate from the ventral region (high *vox*/*vent* and high BMP) to the dorsal region (low *vox*/*vent* and low BMP) and dynamic changes to cell properties to autonomously occur during migration.

Purpose of the present work

In this thesis, I present two proteins related to different signalling pathways (Angiomotin-like 2 for FGF signalling and FrzA for Wnt signalling). They were isolated by two different commonly used methods (Angiomotin-like 2 by microarray and FrzA by large-scale *in situ* hybridization screening). I use the zebrafish as a model for both studies.

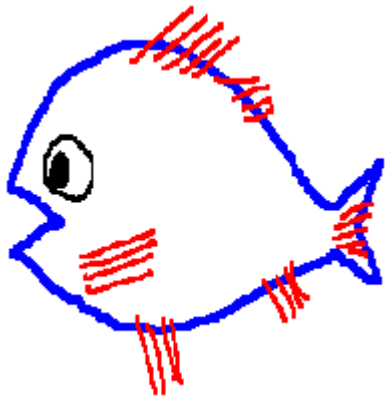
First, previous work on Angiomotin-like 2 was mainly on the mouse and using cell culture and only focused on cell migration. In the present study, I use the zebrafish model to investigate the role of angiomotin like-2 both on cell migration and on patterning.

Second, previous work on FrzA (sFRP1) was performed on the mouse, *Xenopus,* or late-stage zebrafish embryos. The role of FrzA on zebrafish early patterning is still unknown. The present study provides a clearer mechanism for FrzA on Wnt signalling inhibition which was unexpected.

To more easily understand each part of the thesis, I provide a short and closely related introduction at the beginning of each part, followed by the discussion. In the final discussion, I present possible links between these two signals and draw a connection between cell migration and identity.

Part II

The role of Angiomotin like 2 in early zebrafish development



A. Introduction

1. Angiomotin like 2 was found to be a downstream target of FGF signalling.

This project began with cooperation from Anming Meng's group at Tsinghua University, Beijing, China. From a complementary cDNA microarray approach we identified a FGF signalling downstream target gene called Angiomotin like 2 (Amotl2) (Huang et al., 2007). Amotl2, which is the founding member of the Motin family, was characterized as a binding partner of angiostatin by a yeast two-hybrid system; angiostatin is an inhibitor of angiogenesis and cell migration (Troyanovsky et al., 2001).

The open reading frames of Motins have a common in-frame stop codon in the 5' region, and the actual reading frame was deemed to start at the first downstream ATG (Fig. II-1). Motins share approximately 250 residues consisting of a putative coiled-coil domains and another shorter coiled-coil structure further toward to the C-terminus (Fig II-2). When Lars's group further checked the splicing site in the coiled-coil domain among three human motins (angiomotin, angiomotin like 1 (Amotl1), and Amotl2), they found that the three motins share five almost completely conserved splicing sites on their coiled-coil domains. Angiomotin and Amotl1 even share three additional splicing sites in this domain. This indicates that these three proteins have a common evolutionary origin but Amotl2 is an evolutionary outgroup in relation to angiomotin and Amotl1 (Fig II-2, (Bratt et al., 2002)). The coiled-coil domain possibly mediates protein-protein interactions. For example, the coiled-coil domain of angiomotin can bind to Rich1, a Cdc42-selective GTPase-activating protein (GAP) that inhibits Cdc42 GTPase activity through promoting nucleotide hydrolysis and is necessary for angiomotin localization to apical membranes (Wells et al., 2006). There is also another potential domain shared

by these three angiomotins, the PDZ binding domain. The PDZ binding domain is located on the C-terminus of angiomotins and can bind to the C-terminus of other proteins. For example, the PDZ binding domain of Angiomotin is responsible for Angiomotin binding to another tight junction protein, Patj. This interaction through the PDZ binding domain is responsible for angiomotin targeting of tight junctions (Wells et al., 2006). Between the N-terminal part and the coiled-coil domain of Amotl1 and Amotl2, there is also a domain that contains the 12 conserved glutamine residues, known as glutamine-rich domains. In angiomotin, there is another domain, the angiostatin-binding domain, located between the putative coiled-coil domain and the PDZ binding domain. As a result, Amotl1 and Amotl2 likely do not bind angiostatin. In addition, the immunoprecipitation showed that in in vivo situations, angiomotin forms a complex with Amotl1 and Amotl2 (Wells et al., 2006).

To determine angiomotin distribution in the cell, Lars's group showed the distribution of the different angiomotin isoforms (p130 and p80 angiomotin) on the cell membrane. Both the N- and C-terminal parts, including the PDZ binding domain and coiled-coil domain of p-80 and p-130 angiomotin, face the cytoplasm, and only the angiomotin-binding domain is exposed to the extracellular space (Fig. II-3).

2. The role of Angiomotin in vivo and in vitro.

The study of the function of angiomotin in vivo focused on cell migration, either on visceral endoderm (VE) movements in mouse embryos (Shimono and Behringer, 2003) or on endothelial cell migration (Levchenko et al., 2003). VE movement from amot mutant mouse embryos was determined by VE-specific marker staining or by DiI labeling, then the embryos were cultured in vitro. All results showed that visceral endoderm migration was seriously

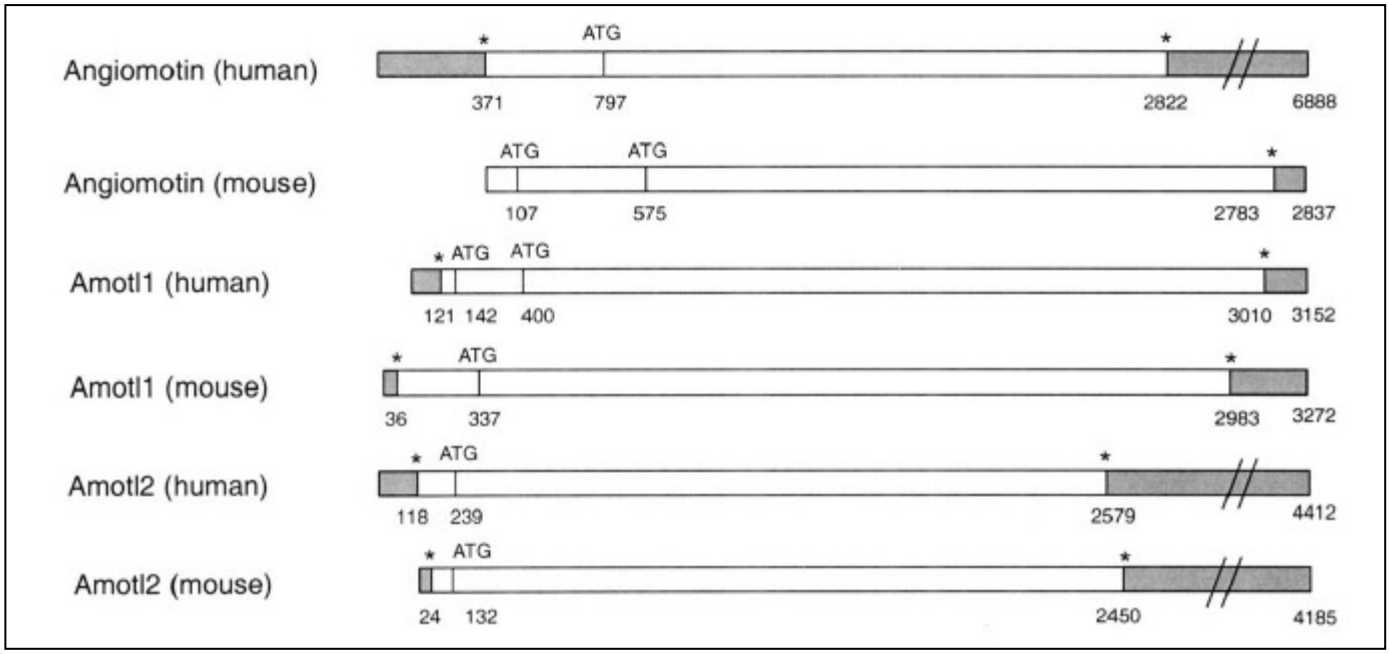


Figure II-1: Open reading frames of angiomotin, amotl1 and amotl2 from human and mouse. Sequences were identified by sequencing and assembly of data available in GenBank. White, open reading frame, gray, non-open reading frame, *, stop codon (Photograph from Bratt et al., 2002).

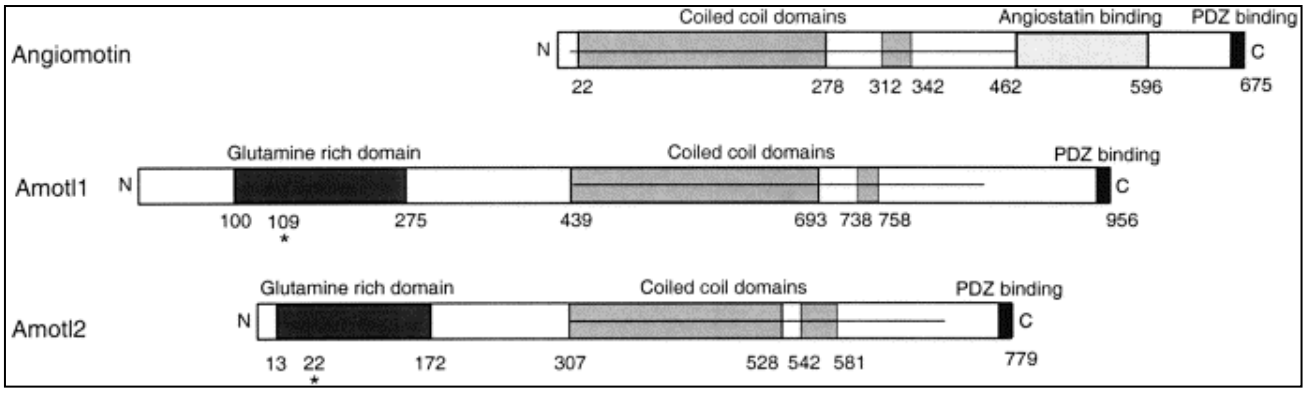


Figure II-2: Domain architecture of human angiomotin, Amotl1 and Amotl2. Numbers indicate residues numbered from the N-terminus. *, conserved putative tyrosine phosphorylation site. Among motins, it is likely that approximately 250 residues forms a coiled-coil domain where exists in the N-terminal of Angiomotin or in the central part of Amotl1 and Amotl2. There is also a shorter coiled-coil structure further towards the C-terminus. Furthermore, the PDZ domain in the carboxy termini of the motins are also conserved and contains hydrophobic residues. In Amotl1 and Amotl2, there have five stretches of 7–20 conserved residues between the N terminus and the coiled-coil domain. Two of these contain a total of 12 conserved glutamine residues and therefore be designated as glutamine rich domains. The angiostatin binding domain of angiomotin, a sequence rich in alanine and proline, was identified by the yeast two-hybrid screen (Troyanovsky et al., 2001). This domain is located between the putative coiled-coil and the PDZ-binding domains of angiomotin. This domain is present in mouse angiomotin but not in Amotl1 and Amotl2. Therefore it is not likely that Amotl1 and Amotl2 bind to angiostatin. (Photograph from Bratt et al., 2002).

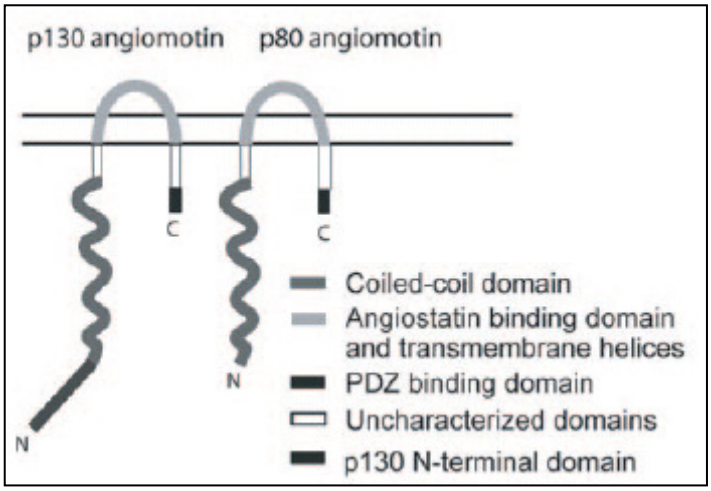


Figure II-3: The suggested model for the topology of p80 and p130 angiomotin (Photograph from Bratt et al., 2005). From the result of different antibodies labeling that against different domains of angiomotin suggest that the N-terminal (include P130 N-terminal domain and coiled-coil domain) and the C-terminal (include PDZ domain) of angiomotin are intracellular and that the hydrophobic regions flanking the angiostatin binding domain (ABD) can form two transmembrane helices and leaving the central part of this domain in the extracellular space.

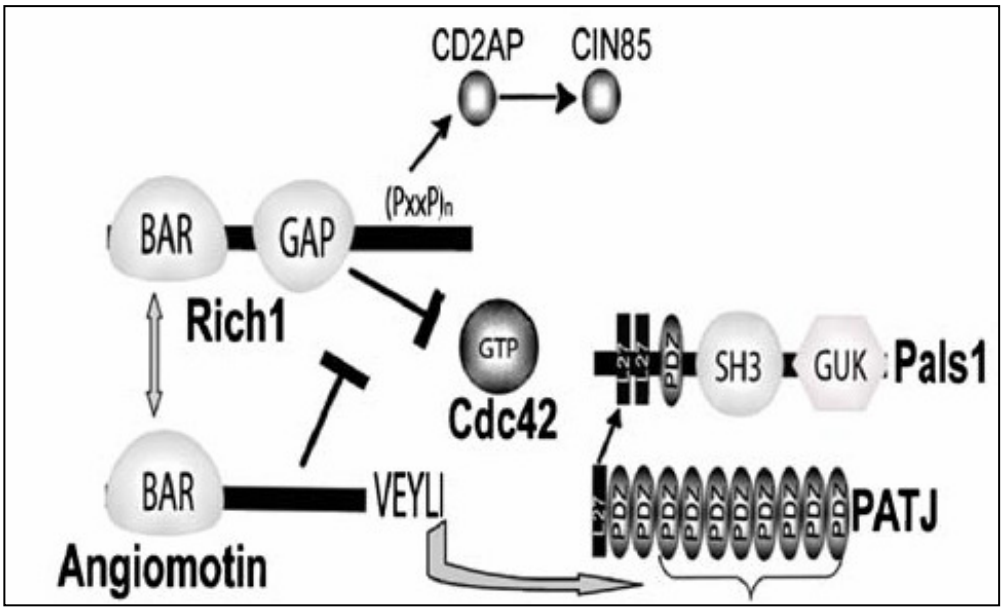


Figure II-4: A depiction of the domain architectures and proposed functional interactions of Rich1, Amot, Pals1, Patj, and Cdc42 (Photograph from Well et al., 2006). The experiment data indicate that Amot is a scaffold that recognizes Patj through its C-terminal PDZ binding motif and also binds Rich1 through a mutual BAR-domain/Coiled-coil-domain interaction and suppresses the ability of Rich1 to reduce the fraction of Cdc42 bound to GTP. In addition, Patj and Pals1 also interact directly through heterodimerization of their N-terminal L27 domains (Roh et al., 2002). Thus, Amot links Rich1 to Patj and may thereby target Rich1 to a subpopulation of Cdc42 involved in maintaining TJ structures.

retarded, and embryos with this delayed phenotype died soon after gastrulation. In addition, the function of angiomotin was specific to the VE and further confirms its expression in vivo. In another transgenic mouse experiment, Lars's group used a PDZ binding domain deletion construct driven by an endothelial cell-specific promoter (receptor tyrosine kinase, TIE). The results showed that the specific expression of the mutated form of angiomotin in endothelial cells inhibited cell migration into the neuroectoderm and intersomitic regions resulting in death at embryonic day 9.5. This again shows the importance of the PDZ binding domain to angiomotin and agrees with the domain analysis of the sequence.

The in vitro study of angiomotin used several methods. The first was the tube formation assay which can detect cell migration by endothelial cell morphology in polymerized Matrigel culture. The second was a Boyden chamber assay to observe cells migrating from one chamber to another. The results showed that cells transfected with the angiomotin deleted from its PDZ binding domain could not form tubes and lost the chemotactic response to known endothelial cell migration induction factors (bFGF, VEGF, and LPA) (Levchenko et al., 2003, Aase, 2007 #339). Finally p130-angiomotin could not promote cell migration and did not respond to angiostatin as p80-angiomotin does. Nevertheless, p130-angiomotin did interact with F-actin through its N-terminal extension and affected the cell shape (Ernkvist et al., 2006). In addition overexpression of angiomotin disrupted tight junction structure whereas angiomotin knockdown did not (Wells et al., 2006). Finally, the overexpression of angiomotin suppresses the ability of Rich1 to reduce the fraction of Cdc42 bound to GTP and ultimately activates Cdc42 GTPase. But AmotI1 has no such direct function in Rich1 suppression (Fig. II-4).

Taking all these data together, most research on angiomotin has been on angiomotin alone or some on Amotl1, while studies on the function of Amotl2 are still scarce. Most of the results on angiomotin are related to cell migration and cell junctions. No data is available on the role of amotl2 on patterning of the early embryo. Therefore I attempted to determine the function of Amotl2 using zebrafish embryos as a model system to study in vivo cell migration mechanisms as well as its role during early embryo pattern formation.

B. Specific experimental procedures

1. Local injection in 64 cell stage embryos

The embryos are injected randomly in one blastomere at the margin at the 64 cell stage with morpholino (MO) and/or RNA coding for the GFP (Fig. II-5A). Embryos are allowed to develop until the shield stage, then they are sorted out according to the position of the labelled clone of cells under a fluorescence dissecting microscope (Fig. II-5B). After the selection, embryos are allowed to develop until 12 or 24 hours in a 0.3 x Danieau buffer (Fig. II-5C). For taking pictures, embryos older than 24 hour stage were anesthetized using Tricaine (Sigma A-5040) then mounted in a 4 -coverslide bridges viewing chambers with 2 % methylcellulose as described in the zebrafish book (Westerfield, 2000).The photos are taken using a Coolsnap CCD camera on a fluorescence macroscope (Leica MZFLIII).

2. Observation the cell lamellipodia in live embryo

The embryos are injected randomly in one blastomere at the 64 cell stage at a position at 45 degree from the margin in the lateral side. At the 70% epiboly stage, the embryos are mounted on a 4-coverslide bridges viewing chamber at 37 degree in 1.2% low-melting point agarose then they are observed using a confocal microscope with the GFP channel (Leica TCS SP2).

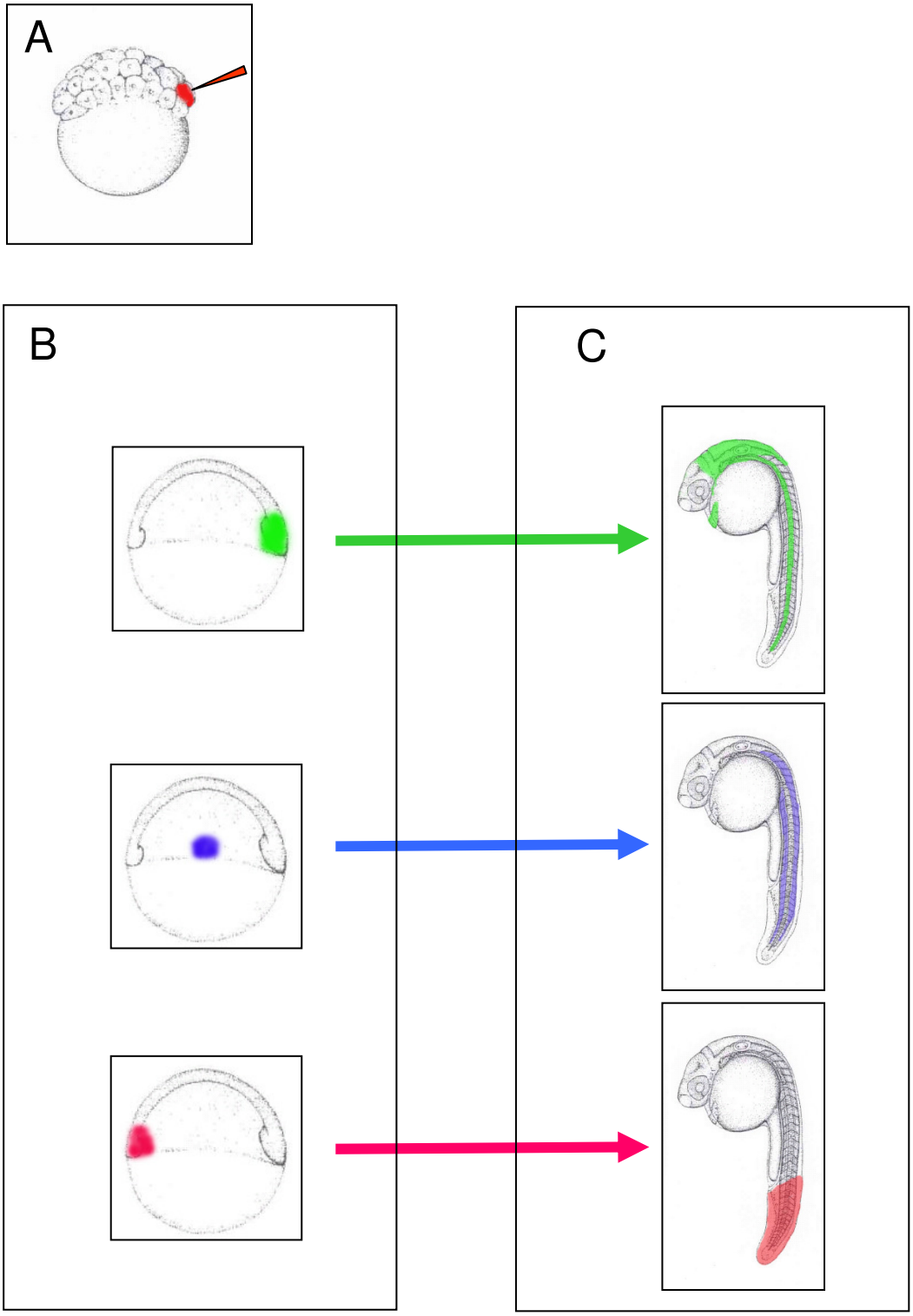


Figure II-5: The Local injection on 64 cell stage embryo. The injection is randomly injected the GFP RNA into one marginal cell at the 64 cells stage (A). The injected embryos development until shield stage (B) then separated into three groups include dorsal (upper), lateral (middle) and ventral (Lower) groups depends on the position of injected cells compare to the shield position which is the dorsal side. All the embryos dorsal is to the right, ventral to the left. The separated embryos continue to development until 24 hours then check the position of labelled cells (C). In the GFP RNA injection alone, the Dorsal (green) labelled cells migrate into posterior brain, notochord and hatching gland. The Lateral (Blue) labelled cells migrate into trunk and and Ventral (Red) labelled cells migrate into tail region.

C. Results

1. The relationship between the Angiomotin like 2 and the zebrafish cell migration.

a. Article: "Amotl2 is essential for cell movements in zebrafish embryo and regulates c-Src translocation".

Development 134, 979-988

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Amotl2 is essential for cell movements in zebrafish embryo and regulates c-Src translocation

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b. Link between Amotl2 and BMP on the cell migration ability

We already showed that lost of amotl2 will impair cell movements either on the whole embryo or in a local group of cells. The next question is by which molecular mechanisms this cell migration can be affected. Angiomotin like 2 is a downstream target of the FGF signalling and FGF acting through the FGFR1 and the ras-MAP kinase pathway can negatively regulate BMP gene expression (Furthauer et al., 2004). In addition, both BMP and FGF signalling are known to be involved in the regulation of cell movement (Myers et al., 2002a; Thisse and Thisse, 2005; von der Hardt et al., 2007). This is why I first investigated the possible link between BMP signalling and Amotl2.

BMP gene expression disappears after amotl2 knock down, (see the next section of amotl2 result on the patterning, Fig. II-9G). This was unexpected because the stimulation of FGF results in a repression of BMP transcription, the loss of function of one downstream element of the FGF signalling pathway should not have a negative effect on BMP expression. However, BMP signalling is downregulated in amotl2 morphant and I hypothesized that the initial defect in cell movement in amotl2 loss of function condition may result from this impairment in BMP signalling.

To experimentally address this question, I tried to inhibit the BMP signalling in cell autonomous conditions. To do this, I injected a truncated BMP receptor (TBR) which blocks the BMP signalling cell autonomously (Frisch and Wright, 1998) in one blastomere at the 128 cell stage. In parallel, to compare the effect of local BMP loss of function with Amotl2 loss of function I performed the same experiment in the same conditions by injecting amotl2 antisense MO. To perform this experiment I choose to inject the cells at 45 degree from the margin (Fig. II-6A) because in that part of the embryo other signalling

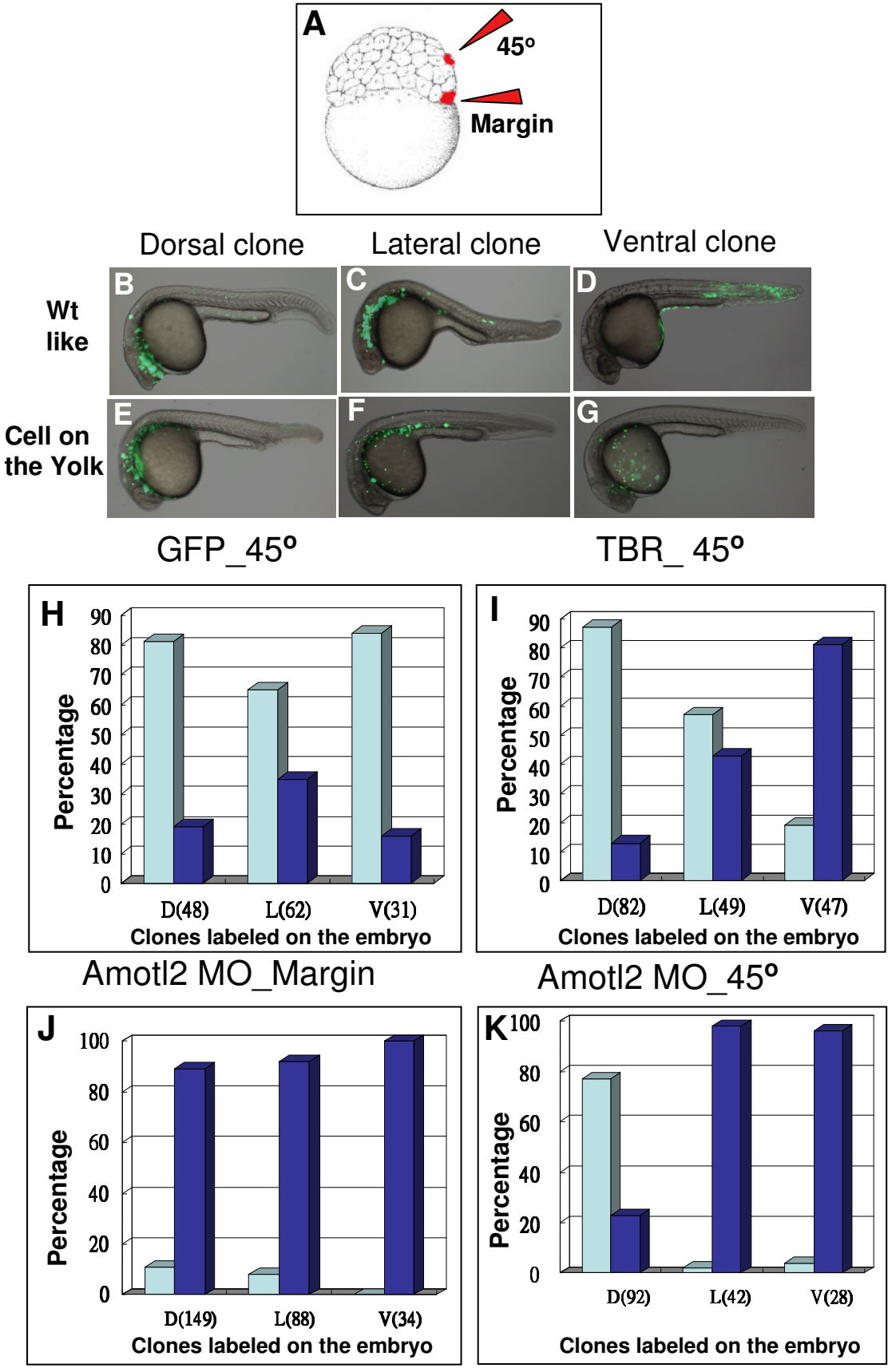


Figure II-6: The local injection of GFP (Green Fluorescence Protein) or TBR (Truncated BMP receptor) or Amotl2 antisense MO in one cell on the margin or 45 degree compare to the blastodisc at 128 cells stage. (A) The position of random injection, (B-D, E-G) The 24 hours injected embryos which the injected cells were separated at shield stage into dorsal (D), lateral (L) and ventral (V) clones showed either the cells still on the yolk (E, F, G and dark blue columns in the paragraph) or migration as wild type (B, C, D and light columns in the paragraph). In all the photographs (H-K) the injected material and position are labeled above. For each injection the embryos were calculated (The total embryo numbers are labeled in the bracket next to different clone indicators in the horizontal label) and divided into the percentage at 24 hours.

pathways (like FGF, Nodal and Wnt) are less active (Fig. I-9) and are more likely in conditions allow seeing the effect on the BMP signalling only.

Clones of cells deriving from a blastomere injected with GFP alone at 45 degree from the margin, converge into the embryonic axis (Fig. II-6H). Nevertheless, clones derived from TBR injected blastomere, display an impairement in cell movement. Dorsally located clones migrate as wild-type while lateral clones as well as ventral clones are moderately or strongly affected in their convergence movement toward the embryonic axis (Fig. II-6I). This observation is in good agreement with the physiological BMP activity gradient which decreases from ventral to dorsal. The TBR injected cells which cannot receive BMP signalling will behave as dorsal cells and can behave as the surrounding wild type cells when placed in a low BMP territory (dorsal) but when they are in a high BMP environment (Ventral), they cannot move to the dorsal part of the embryo.

Compared to the amotl2 morphant clone of cells, the cell autonomous inhibition of the BMP signalling pathway leads to weaker phenotype when clones are in a lateral territory and there is a clear ventral to dorsal decrease in the strength of cell movement defects which is not observed for amotl2 loss of function. Nevertheless, the results are similar enough to make the hypothesis that the defect of cell migration in amotl2 may be, at least for part, to a defect in the BMP signalling property or in the ability of cells to receive the BMP signal. To investigate this hypothesis I tried to rescue the amotl2 migration defect by co-injecting amotl2 antisense MO with an active form of Smad1 (Ras-insensitive, (Kretzschmar et al., 1999)) which can activate cell autonomously the BMP signalling pathway. In this condition I never observed any rescue of the convergence movement in clones inactivated by antisense MO injection for Amotl2 (not shown). These results strongly suggest that even

if phenotypes of BMP and Amotl2 loss of function are similar, the impairment of cell movement in Amotl2 morphant is not due to a defect of BMP signalling for these cells migration.

c. Link between Amotl2, Cdc42 , Src and E-cadherin

During my previous experiment I ruled out that the inhibition of cell movement of amotl2 morphants cells was due to their inability to respond to BMP signalling. In order to study the possible cause of this defect I investigate the link between amotl2 and several components known to be implicated in the control of cell movement: the small GTPase Cdc42, Src kinase and the E cadherin. The Cdc42 GTPase which is linked to the cell tight junction and polarity has been shown to be positively regulated by angiomotin through Rich1 GTPase activating proteins ((Wells et al., 2006), Fig. II-4). Therefore, I hypothesized that the Cdc42 activity may be affected after amotl2 knock down and coinjected a constitutively active form of Cdc42 RNA which is defective for the GTP hydrolysis (Cdc42 G12V, (Djiane et al., 2000; Drechsel et al., 1997)) with the amotl2 antisense MO in the whole embryo. Preliminary data showed that the epiboly movement was even more seriously impaired and delayed when the active form of Cdc42 was co-expressed in amotl2 morphants (compare Fig. II-7A, B, C). Therefore, the cell movement cannot be rescued by the Cdc42 signalling. To confirm this result I also performed local marginal cell co-injection at the 64 cell stage. In these conditions the injected clones stayed on the yolk and did not move into the trunk (Data not shown).

Then I investigated the interaction between amotl2 and the non-receptor tyrosine kinase Src. Src plays important role on actin cytoskeleton remodeling

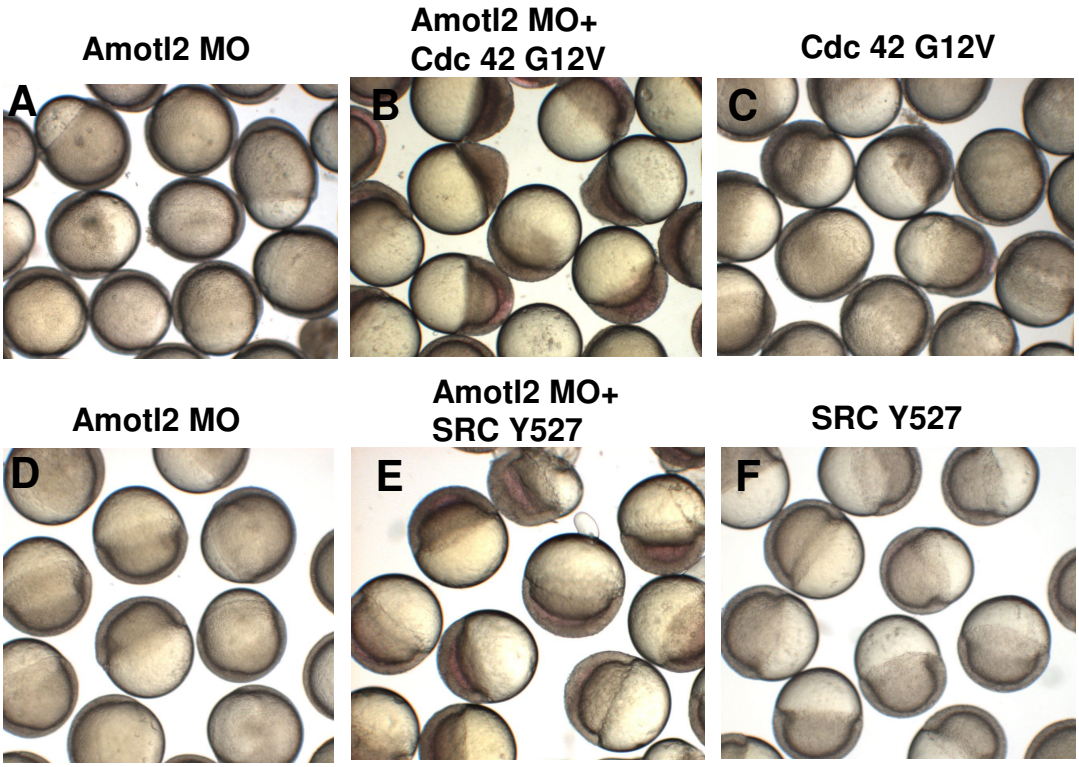


Figure II-7: Rescue experiment for the amotl2 morphant. The rescues experiment were performed by injection in the whole embryo. The combinations are amotl2 antisense MO alone (A, D) or amotl2 antisense MO combine either with Cdc42 G12V RNA (B) or with SRC Y527 RNA (E) or Cdc42 G12 RNA alone (C) or SRC Y527 RNA alone (F). After injection, the embryos were checked by the phenotype at 70% epiboly. The result on the epiboly movement showed that compare to the amotl2 morphant (A, D) or constitutively activated Cdc42 (B) or Src (E) injected embryos, none of the amotl2 morphant clones expressing either the constitutively activated Cdc42 (B) or Src (E) displayed a rescued cell movement.

and cell motility and needs RhoB containing endosome to promote its translocation from perinuclear to the peripheral cell-matrix region (Fincham et al., 1996; Ishizawar and Parsons, 2004). We already showed that lost of function of amotl2 will inhibit the translocation of phosphorylated c-Src from the base to the top of the lamellipodia (Huang et al., 2007). In consequence, cells lacking amotl2 function have less active form of c-Src at their periphery. Therefore I tried to rescue the movement of amotl2 morphant cells by co-injecting a constitutive form of c-Src RNA (Src Y527; (Sandilands et al., 2004)) with amotl2 antisense MO assuming that a strong expression of activated Src activity may rescue the cell migration defect. Unfortunately, in a preliminary experiment none of the amotl2 morphant clones expressing the constitutively activated Src displayed a rescued cell movement (Fig. II-7D-E). It is likely that even a high concentration of activated Src could not passively diffuse in the cell and that amotl2 is absolutely required for the translocation of activated Src at the periphery of the cell. Nevertheless, a study of the precise localization of phosphor-Src in these experimental conditions needs to be done to confirm this last statement.

Because all movements, including epiboly are more seriously delayed when increasing concentration of amotl2 antisense MO are performed (Huang et al., 2007) it is likely that cells in the amotl2 antisense MO injected embryos may be tightly connected to each other. This tight cell connection is obvious in clones derived from a local injection where morphant cells appear to aggregate (Fig. II-8A, B).

A similar phenotype of aggregated cells can also be found in cells lacking BMP signalling through the loss of function of the BMP type I receptor (Alk8) in zebrafish gastrula cell culture (von der Hardt et al., 2007). In this study, authors show that a dominant negative cadherin that affects the Ca^{2+}-mediated cell

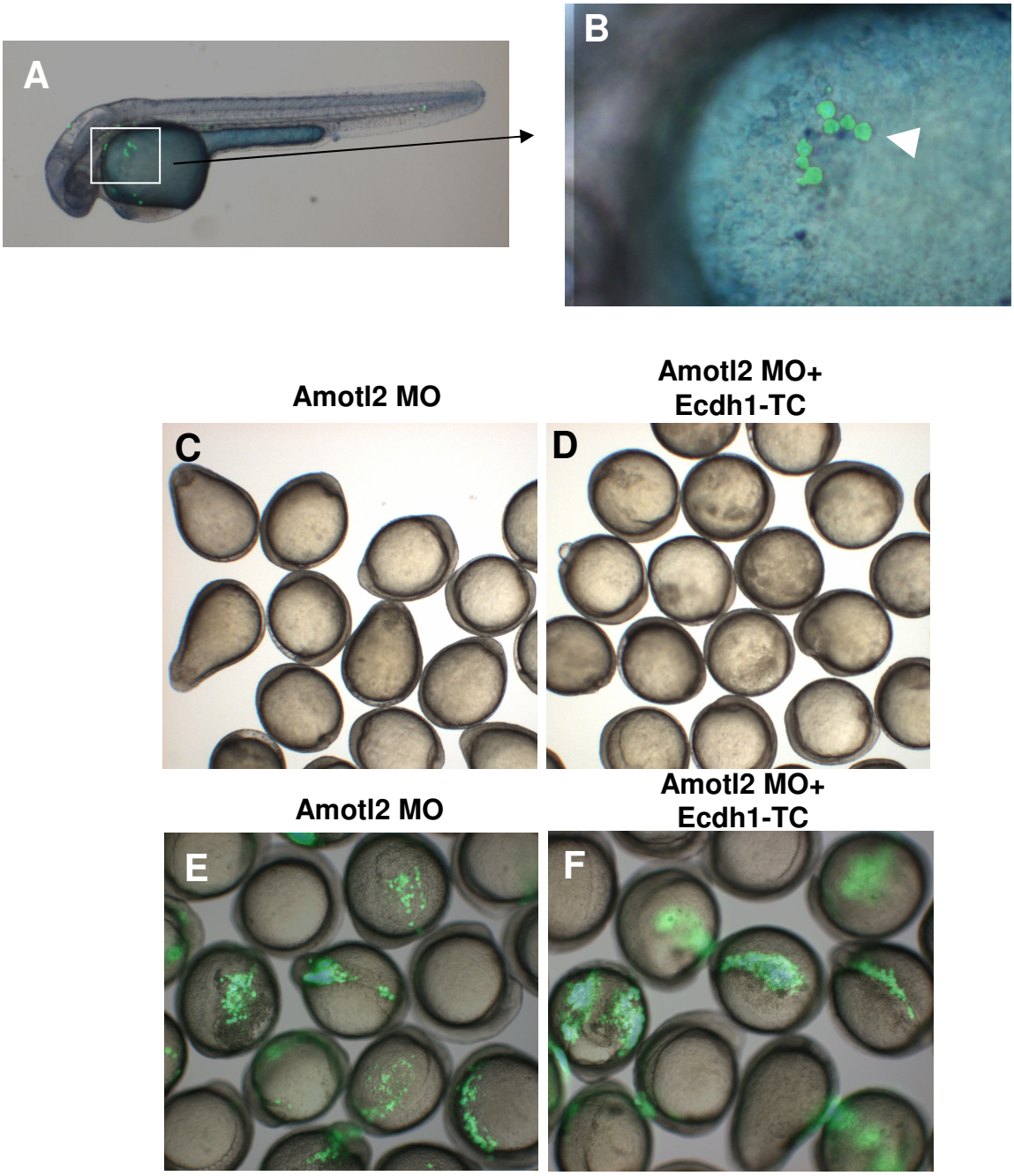


Figure II-8: Rescue experiment for the amotl2 morphant. (A, B) the amotl2 antisense MO injected cell left on the Yolk which have green fluorescence and aggregate in few big cells (indicated by arrow head). The blue background is the Nile blue staining for the apoptosis detection. The rescues experiment were performed by injection in the whole embryo (C, D) or in one cell at 64 cell stage (E, F). The combinations are amotl2 antisense MO alone (C, E) or with Truncated form E-cadherin RNA (Ecdh1-TC, D, F). After injection, the embryos were checked by the phenotype at 70% epiboly (C, D) or by the fluorescence at somitogenesis stage (E, F). The elongated shape of the embryo due to amotl2 antisense MO injection may be rescued by Ecdh1-TC RNA co-injection (C, D). However, in the preliminary local co-injection experiment, the cell movement does not seem to be rescued (E, F).

adhesion can rescue this aggregation phenotype. However, the link between BMP signalling and cadherin is still not clear and both pathways can function in parallel. Although the function of amotl2 does not seem to be linked to the BMP signalling, the defect on the cell adhesion is similar. This is why I investigated the relation between amotl2 and cadherin. In a preliminary experiment I used the truncated from of epithelial – cadherin (ECdh1-TC) to try to rescue the lost of amotl2 phenotype either in the whole embryo or after local injection of antisense MO. In these conditions I observed that the elongated shape of the embryo due to amotl2 antisense MO injection may be rescued by Ecdh1-TC RNA co-injection (compare Fig. II-8C and D, the Ecdh1-TC injected alone gave wild type like embryos - not shown). However, in this preliminary experiment, for local co-injection, the cell movement defect does not seem to be rescued (Fig. II-8E and F). More experimental conditions including series of various concentrations and combinations for both amotl2 and Ecdh1-TC need to be tested before reaching a final conclusion.

2. Angiomotin like 2 and embryonic pattern formation.

a. Amotl2 is necessary for correct patterning of the embryo.

In addition to the alterations on cell migration my analysis by in situ hybridization of amotl2 morphants revealed strong abnormalities in the patterning of these embryos. After knock down of Amotl2, the expression of molecular markers related to the BMP signalling pathway changed significantly. For example, the expression of the dorsal markers Noggin and Chordin, two BMP inhibitors, extended all around the margin (Fig. II-9E, F). Concomitantly, the expression of BMP4 and Bambi were decreased (Fig. II-9G, H).

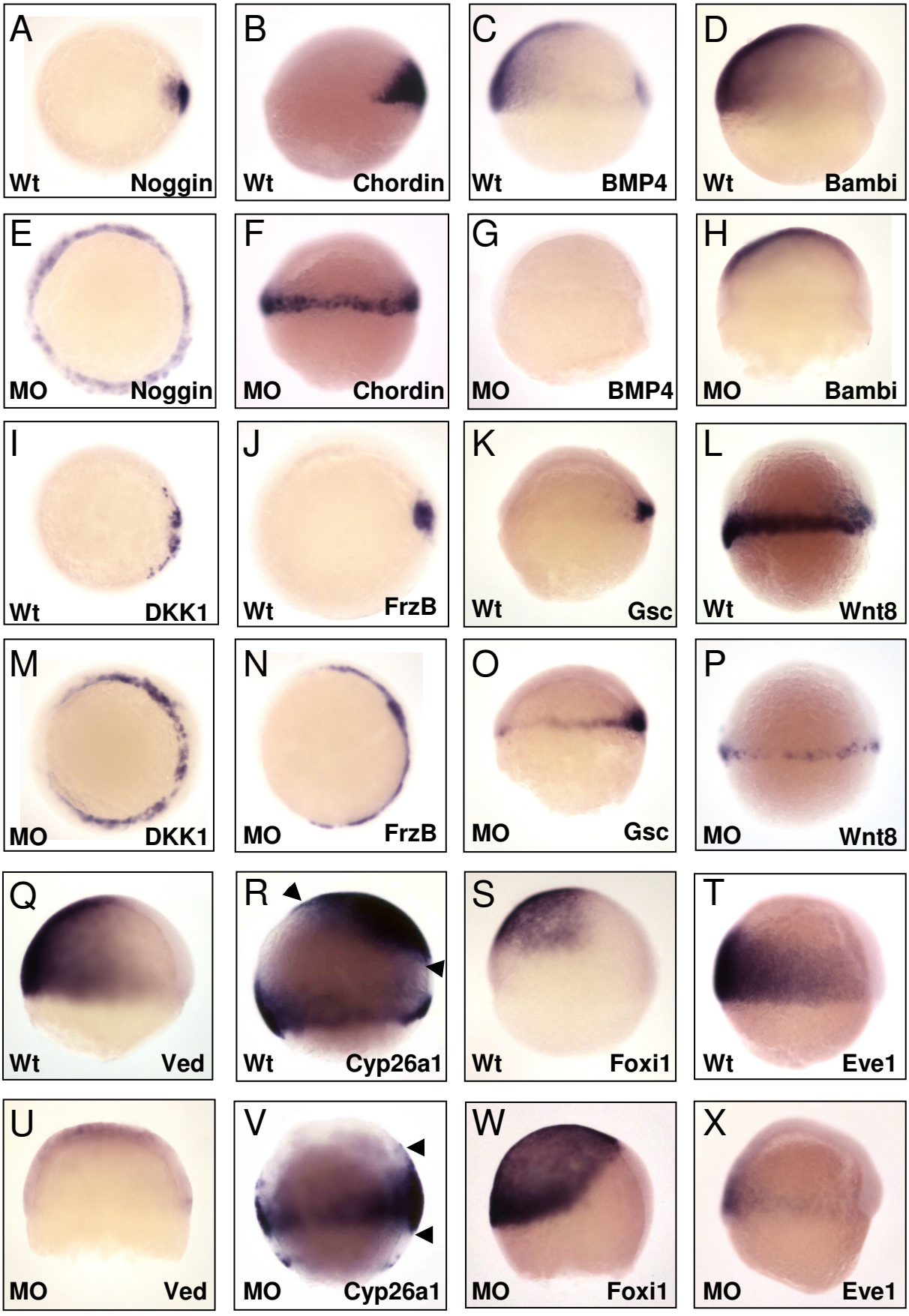


Figure II-9: The genes expression was altered after knocked down the Amotl2 gene. Compare the wt (A-D,I-L,Q-T) and the Amotl2 antisense MO injected embryos (E-H, M-P,U-X) with different markers. Noggin (A, E), Chordin (B,F), BMP4(C,G), Bambi (D, H), DKK1(I, M), FrzB (J, N), Goosecoid (K, O), Wnt8(L, P), Ved (Q,U), Cyp26(R, V), Foxi (S, W), Eve1(T,X). Shield stages (A-Q, T, U, X), 80% epiboly (R, S, V, W). All embryos are in the lateral view except A, E, I, J, M, N are animal pole view. Dorsal side towards right. The arrowheads indicated the dorsal anterior and the posterior terminal of the Cyp26a1 expression (R,V).

Similar results were also observed using molecular markers genes known to be involved in the regulation of the Wnt signalling pathway. The expression of the Wnt dorsal inhibitors includes Dickkopf 1(DKK1) and FrzB as well as the dorsal transcription factor Goosecoid (Gsc), that has been shown to act as a repressor of Wnt8 expression (Glinka et al., 1998; Seiliez et al., 2006) were examined and shown to extend toward the ventral part of the margin (Fig. II-9M-O). Conversely, expression of the components of the Wnt pathway (Shimizu et al., 2005a), Wnt8 and Ved, expressed ventrally in wild type embryo, strongly decreased (Fig. II-9P) or was almost absent (Fig. II-9U) on the margin.

In the epidermis, the presumptive brain marker Cyp26a1 (cytochrome P450 subfamily XXVIA polypeptide 1) is strongly affected. The presumptive brain region is misplaced and undergoes a 90° turn as indicated by the arrowhead (Fig. II-9R, V). The expression of Foxi1 is also strongly affected and extends both dorsally and vegetally. Finally, the expression of Eve1 decreases after amotl2 knock down (Fig. II-9X).

Altogether these observations demonstrate that in addition to defects in convergence-extension movements, loss of function of amotl2 results in a global change of the embryo patterning.

Indeed, dorsal marginal genes including Noggin, Chordin, DKK1, FrzB and Goosecoid extended toward the ventral side. The expression of the ventral markers BMP4, Bambi and Ved are strongly decreased or almost abolished. These data show that in amotl2 morphants the whole patterning has changed. In the ectodermal layer the territory appears misplaced and a 90° rotation of the neurectoderm is observed with the remaining part of the ectoderm expressing an epidermal marker. The expressions of the various dorsal markers analyzed all extend all around the margin. Interestingly, all marginal cells expressed markers corresponding to the territory that fail to undergo

convergence movement. This raises the question of what could be the first and main event occurring in the embryo, a change in the patterning, that will result in an alteration of cell movement, or a change in the cell movement ability that may affect or control the patterning of the embryo.

b. Amotl2 knock down affects patterning of the embryo before the onset of cell migration.

Because at blastula stage the patterning of the embryo is established at a time where the convergence movement has not started yet, I made the hypothesis that the effect on patterning is preceding the effect on cell movement.

Because injection of MOs frequently results in a delay in development the staging of injected embryos is not easy. To prevent this problem I performed in situ hybridization with chordin on embryos fixed every 30 minutes starting at 30% epiboly that is during late blastula stage and before the beginning of convergence movement. Since at 30% epiboly only the radial intercalation cell movement occurs (Fig. I-3A), while the convergence movement would not be observed before the onset of gastrulation, I choose this stage as the starting point for the observation of different molecular markers.

I used a mild concentration amotl2 antisense MO (3 ng) in order to avoid an early lethality (before the blastula stage) and I checked the expression of the dorsal markers Chordin and Goosecoid as well as the ventral markers BMP4, Ved, Eve1 and Bambi to observe the overall changes in the patterning of the embryo that would be directly linked to the amotl2 lost of function.

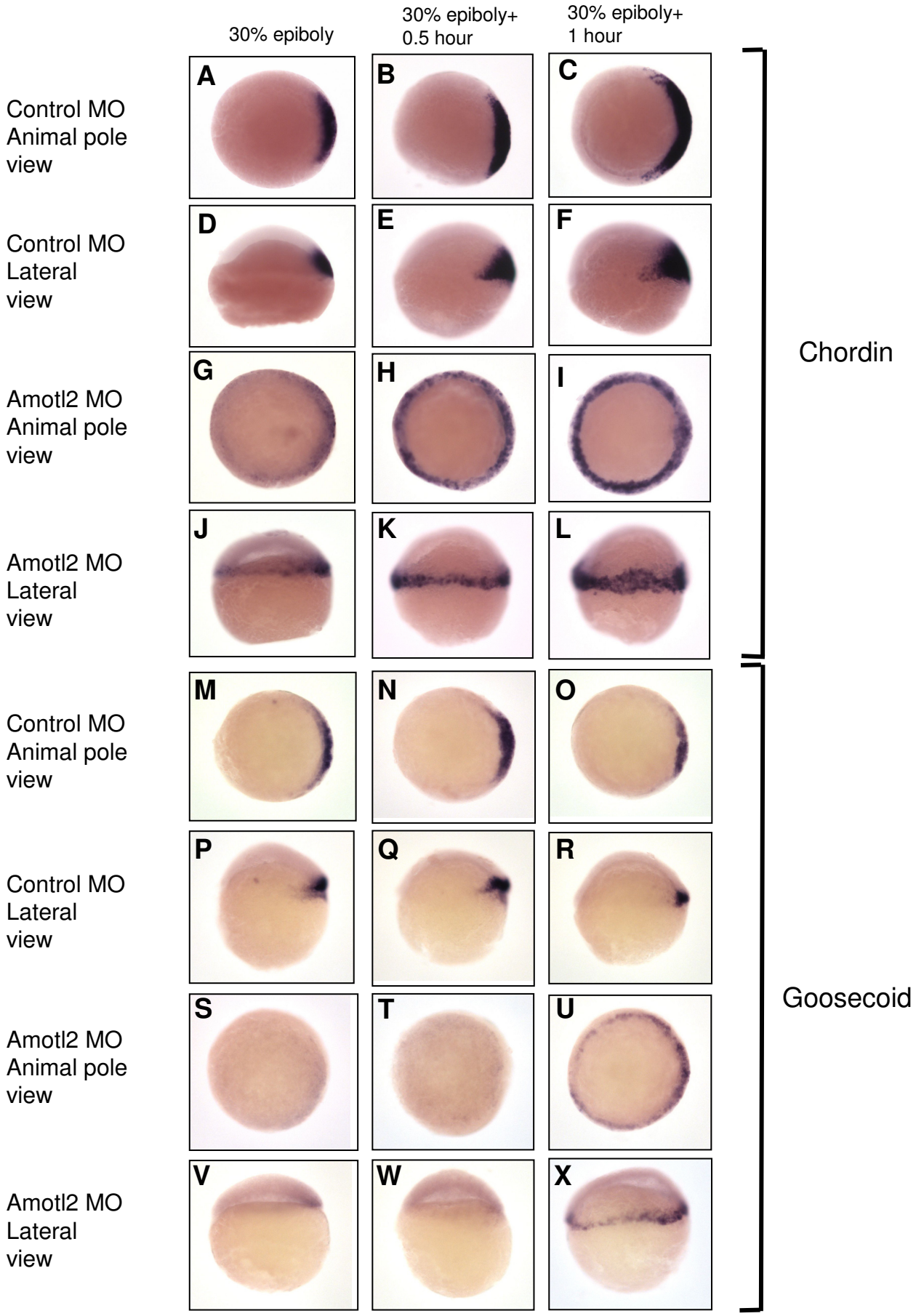


Figure II-10: The different time course of Chordin and Goosecoid expression in the Amotl2 antisense or sense (control) MO injection embryos. (A-L) Chordin in situ hybridization, (M-X) Goosecoid in situ hybridization. (A-F, M-R) control Amotl2 sense MO injection, (G-L, S-X) Amotl2 antisense MO injection. Three columns indicate different sampling time, (A, D, G, J, M, P, S, V) 30% epiboly, (B, E, H, K, N, Q, T, W) 30%+0.5 hours, (C, F, I, L, O, R, U, X) 30%+1 hours. All the embryos are dorsal to the right, (A-C, G-I, M-O, S-U) Animal pole view, (D-F, J-L, P-R, V-X) Lateral view. After Amotl2 inhibition, the dorsal markers already changed as early as 30 % epiboly and it's before horizontal cell movement (convergence).

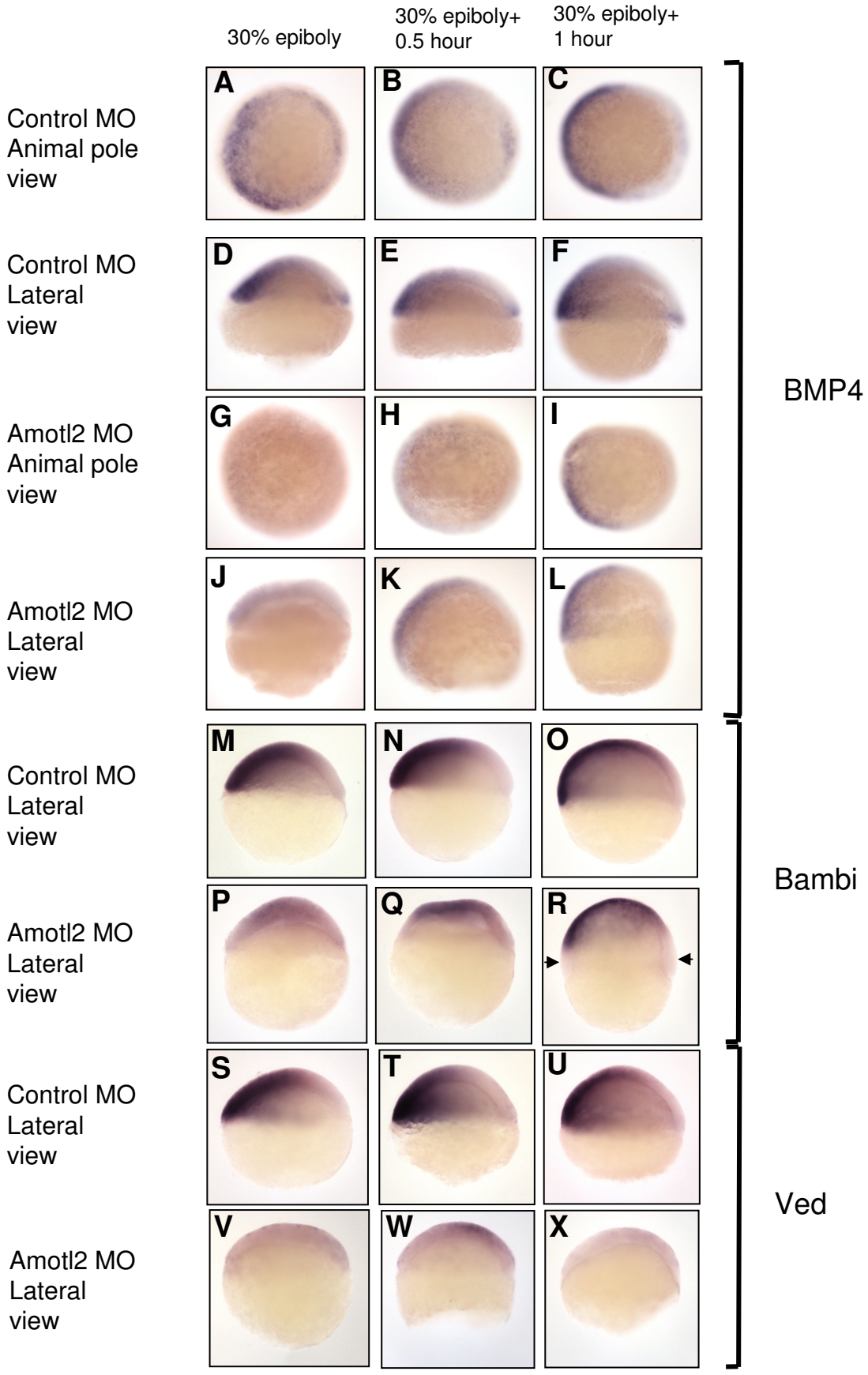


Figure II-11: The different time course of Ventral markers include BMP4, Bambi and Ved expression in the Amotl2 antisense or sense (control) MO injection embryos. (A-F, M-O, S-U) control Amotl2 sense MO injection. (G-L, P-R, V-X) Amotl2 antisense MO injection. Three columns indicate different sampling time, (A, D, G, J, M, P, S, V) 30% epiboly, (B, E, H, K, N, Q, T, W) 30%+0.5 hours, (C, F, I, L, O, R, U, X) 30%+1 hours. All the embryos are dorsal to the right. (A-C, G-I) Animal pole view, the others are all Lateral view. After Amotl2 inhibition, the ventral markers also changed as early as 30 % epiboly.

As early of 30% epiboly, the dorsal marker chordin is already expressed on the ventral side of the embryo, long before the beginning of the convergence movement. Nevertheless, the dorsal expression appears strongly reduced (Fig. II-10G, H, J, K). A similar observation has also been done for Goosecoid (Fig. II-10U, X). At early blastula stage Goosecoid expression is not detected (Fig. II-10S, T, V, W) and when Goosecoid starts to be transcribed, one hour later, its RNA is observed all around the margin.

At the same time, the expression of the ventral maker BMP4 strongly decreased (Fig. II-11G-L) as early as 30% epiboly (Fig. II-11G, J). A similar result is also observed using Bambi, Ved and Eve1 (Fig. II-11P, V, D1). For Bambi expression, the disappearance of transcripts is restricted to the ventral margin, the animal pole expression being maintain (Fig. II-11R, indicated by arrow).

Taking all these data together I can conclude that the amotl2 knock down affects the patterning of the embryo independently from cell movement. These defects are specific of the blastula margin and they may be related to the β-catenin signalling since the wnt8 expression is strongly diminished in the amotl2 morphant. Following this hypothesis, these modifications of the early patterning of the embryo may indicate that the decrease of zygotic Wnt8 signalling in amotl2 knock down results in a lost of the ability to inhibit the dorsal genes at the ventral margin and therefore to maintain the ventral gene expression.

c. Amotl2 loss of function in clones of marginal or animal pole cells

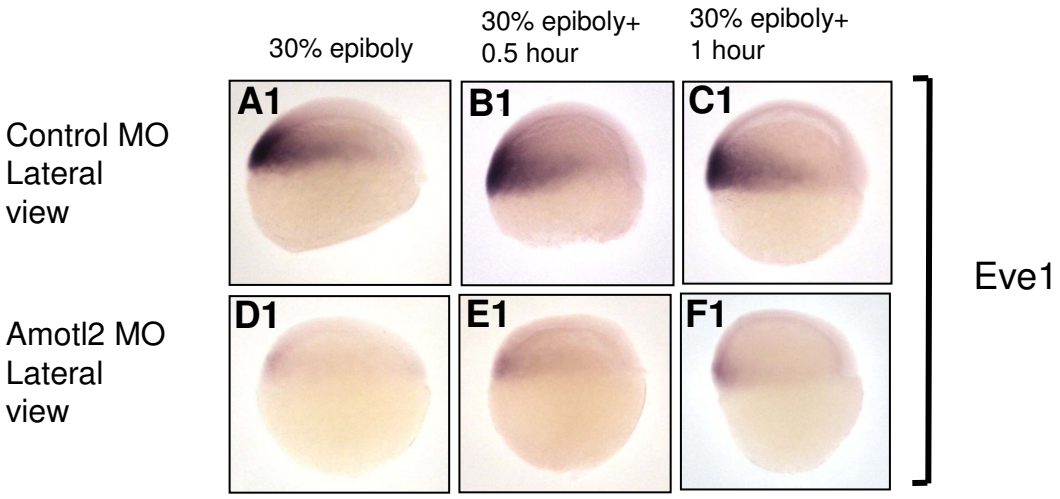


Figure II-11 continue : The different time course of Ventral markers Eve1 expression in the Amotl2 sense (control) or antisense MO injected embryos. (A1-C1) Control MO injection. (D1-F1) Amotl2 antisense MO injection. Three columns indicate different sampling time, (A1, D1) 30% epiboly, (B1, E1) 30%+0.5 hours, (C1, F1) 30%+1 hours. All the embryos are dorsal to the right and are all Lateral view.

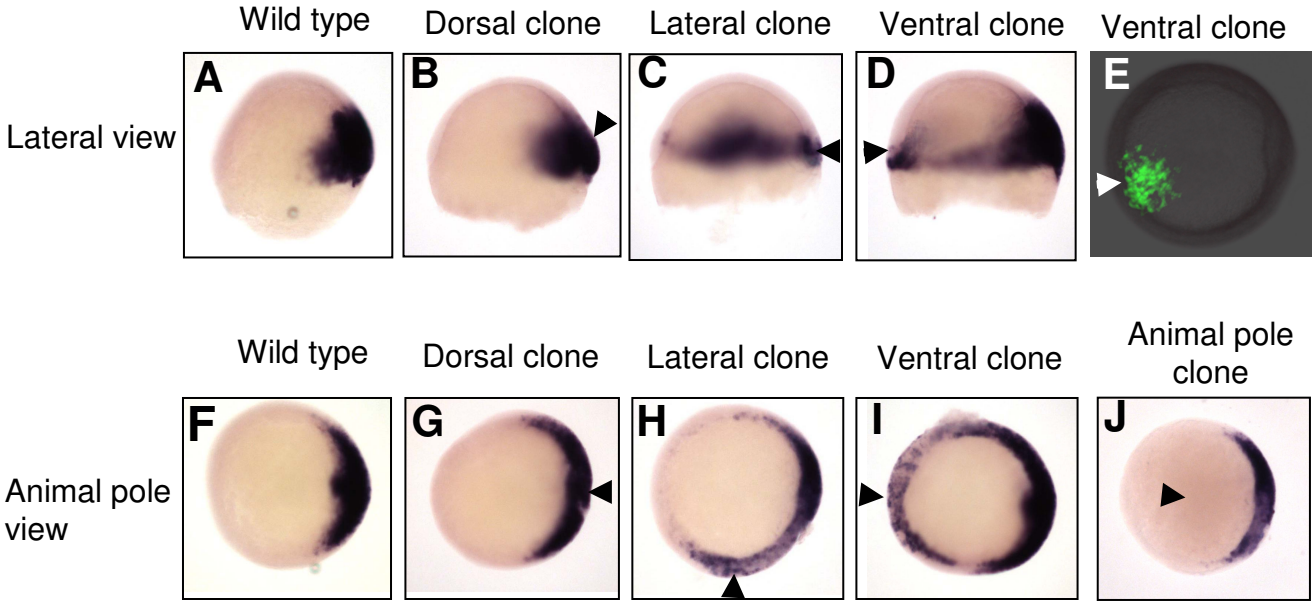


Figure II-12: The Chordin expression at shield stage in the Amotl2 antisense MO local injection embryos. All the local injection are with Amol2 antisense MO plus GFP RNA at one cell in 64 cells stage. (A, B, D) Lateral view, (C) Dorsal view (E-J) Animal pole view. The position of injected clones are above each picture. All the embryos were sampling at the shield stage and dorsal to the right except (C) is front view. The arrow head indicate the position of injected group of cells. The GFP labeled cells in (E) indicate the size of the group of cells at shield stage after injection and compare to the region of chordin expression in (I). Ectopic expression of chordin is detected when the clones of Amotl2 morphant cells are either at the lateral or the ventral margin (C, D, H, I) but not animal pole (J) at the shield stage.

To test the hypothesis mentioned above, I injected a fluorescenlty labelled amotl2 antisense MO into marginal or animal pole blastomere at the 64 cell stage and the embryos have been sorted out at the shield stage based on the localisation of the clone in dorsal, lateral, ventral position at the margin or at animal pole and checked the expression of genes that can be repressed by zygotic Wnt8 signalling. For this experiment I selected Chordin because it is a gene that can be transcriptionally repressed by zygotic Wnt 8 signalling downstream target genes *vent* and *vox* (Imai et al., 2001; Shimizu et al., 2002).

Ectopic expression of chordin is detected when the clones of Amotl2 morphant cells are either at the lateral or the ventral margin at the shield stage (Fig. II-12C, D, H, I, arrow head). The induced expression of chordin is only observed at the margin and I cannot detect chordin expression when the Amotl2 morphants cells are located at the animal pole (Fig. II-12J). In conclusion, the induction of chordin is only observed in territories overlapping or neighbouring the territories of Wnt8 expression. This strongly suggests that this induction of chordin may result from a local inhibition of Wnt8 that will result in a lack of induction of the chordin transcriptional inhibitors Vent and Vox.

In addition, if this hypothesis is true, then the induction of chordin is likely not to be cell autonomous in the clone of Amotl2 morphant cells. The lack of Wnt8, which is a secreted factor, should result in a lack of inhibition of chordin in a broader domain. I checked the cell autonomy of chordin induction in the clone of morphant cells by comparing the size of the clones before fixation (Fig. II-12E) and the size of the chordin expression domain after in situ hybridization (compare Fig. I and E which indicate the size of the clone). In all cases, the clone of chordin expressing cells encompass a broader territory than the

fluorescent clone but is restricted to the Wnt8 expression domain at the gastrula margin.

However, the suggestion that lost of amotl2 function will affect activity or secretion of Wnt8 requires more experimental evidence to be established.

d. Link between Angiomotin like 2 and Wnt 8 secretion

In order to have assess the effect of the lost of amotl2 function on the Wnt 8 secretion I performed an in vivo analysis with a GFP tagged Wnt8 protein. I injected at the one cell stage either a RNA coding for a Wnt8-GFP fusion protein or a combination of RNA coding for Wnt8-GFP fusion protein with amotl2 antisense MO. Then I observed the localization of the fluorescence of the GFP at the shield stage by using a confocal microscope. To make sure that the Wnt8-GFP fusion protein has the same activity as wild type Wnt8 protein I compared the biological activity of the tagged Wnt8-GFP with a wild type Wnt8 and I observed that the phenotypes induced were identical.

The analysis of the localization of the fluorescence GFP-Wnt8 fusion protein after amotl2 antisense MO knock down reveals that a large number of Wnt8 molecules accumulate in the nucleus (compare Fig II-13A, B and C, D arrow indicate nucleus) suggesting a defect in the proper trafficking and secretion of Wnt8. However, additional experiments are required to quantify the extracellular Wnt8 concentration in both experimental conditions before being able to conclude unambiguously about an inactivation of Wnt8 secretion in amotl2 morphants.

Nevertheless these preliminary -result strongly suggest that change of embryo patterning may due to defect in Wnt8 secretion.

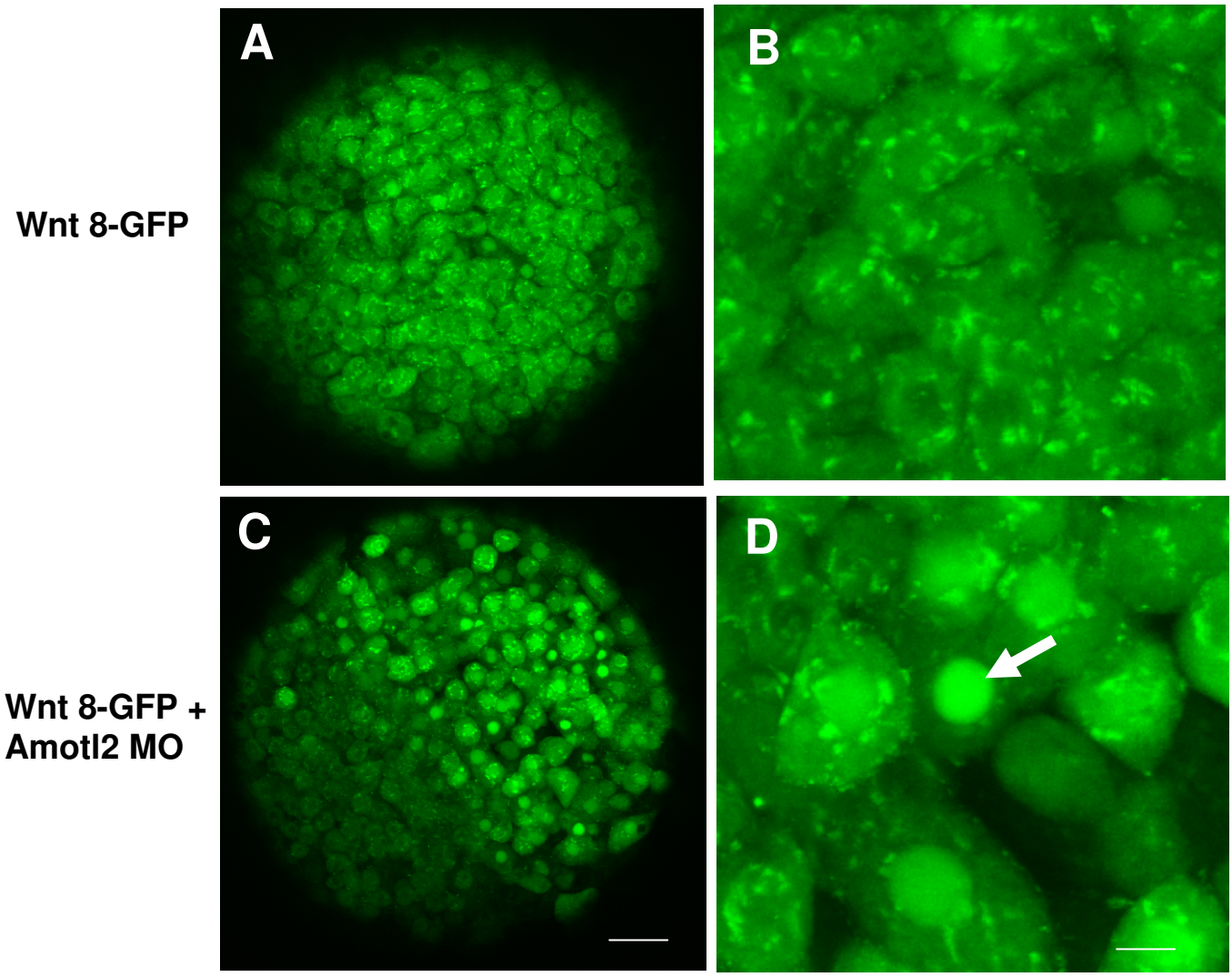


Figure II-13: Expression of Wnt8-GFP in the wild type or Amotl2 knocked down embryo. The embryos were injected with Wnt8-GFP RNA alone or with amotl2 antisense MO in the whole embryo and observe the distribution in the embryo at shield stage under confocal microscope. (A, C) 400X, bar: 60 µm, (B,D) 1600X, bar: 15 µm. The arrow indicates nucleus (D). After amotl2 knock down, a large number of Wnt8 molecules accumulate in the nucleus (C, D**).**

e. Patterning defects following Amotl2 loss of function can be partially rescued by BMP overexpression.

In the previous study, I concentrated my analysis on the effect on the expression of chordin. As this factor is known to be a major regulator of the BMP signalling, I hypothesized that the dorsalization phenotypes observed for partial amotl2 loss of function may be due to the upregulation of chordin as a consequence of a decrease of Wnt8 induced chordin transcriptional inhibitor. To test this hypothesis, I tried to rescue the dorsalization phenotype by injection of RNA coding for BMP4 which can induce ventralization phenotypes (Dale et al., 1992; Jones et al., 1992) and inhibit the dorsal gene expressions by promoting the expression of zygotic Wnt8 downstream gene *Vent/Vox* (Imai et al., 2001; Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000).

I injected embryos at the one cell stage with mild concentration amotl2 antisense MO 1.25 μg alone (this allows the embryos to survive until 24 hours of development) as well as amotl2 antisense MO 1.25 μg plus 10 ng of BMP4 RNA or 10 ng of BMP4 RNA alone and analysed the phenotype at 24 hours. Most (90%) of the amotl2 antisense MO injected embryo showed intact head but the tail was curled due to the decrease of ventral tail tissues (Fig. II-14A, F, white column). However, this dorsalization decreased from 90% to 36% when the BMP RNA was coinjected with Amotl2 antisense MO and formation of ventral tail tissues appeared to be rescued (Fig. II-14C, F, grey column). This result showed that the defects of embryo patterning resulting from loss of amotl2 function can be at least partially rescued by the BMP signalling.

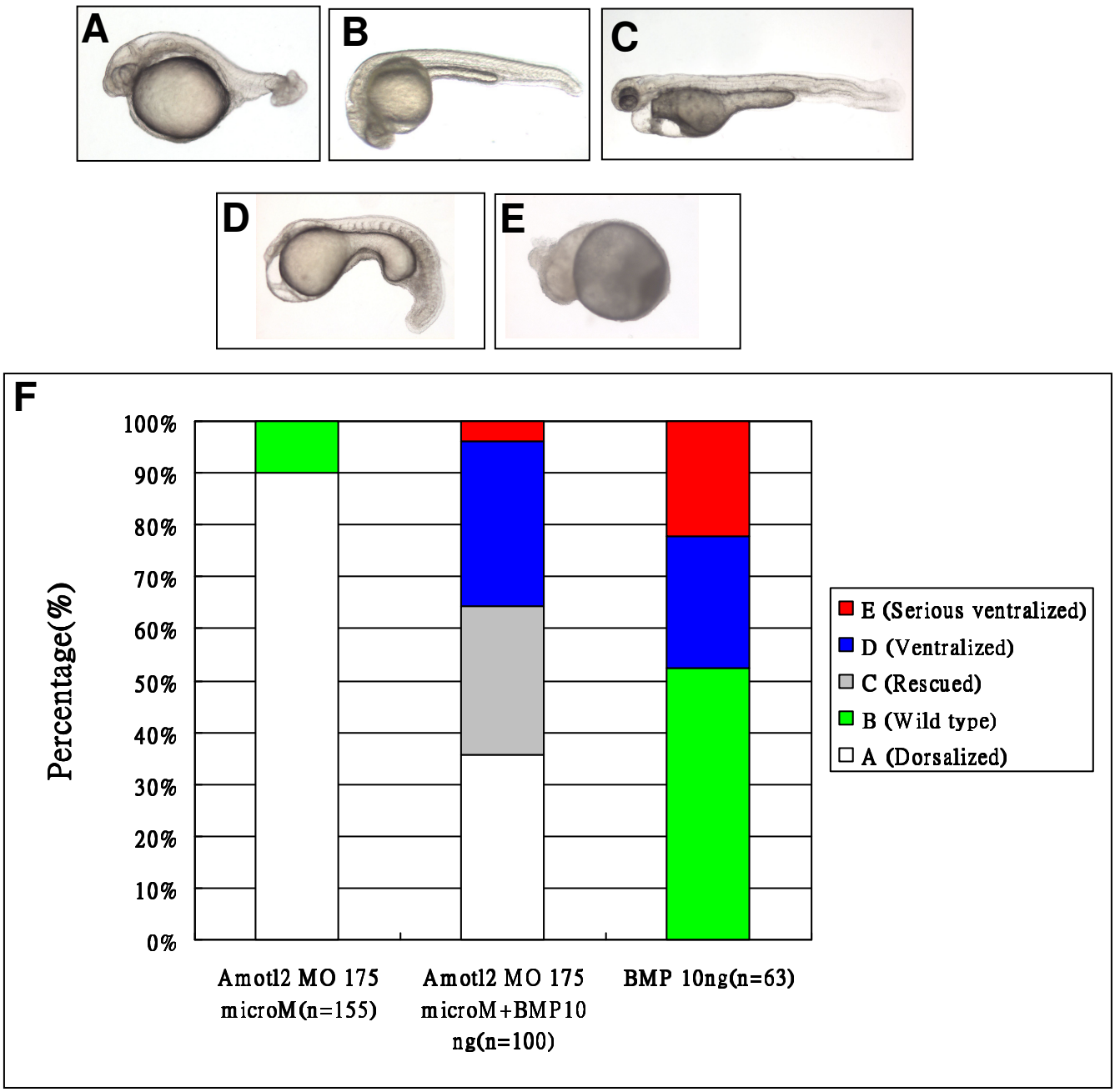


Figure II-14: The rescue of the Amotl2 knocked down embryo by the BMP overexpression. (A) Amotl2 knocked down Dorsalized embryo with intact head but the tail was curled due to the decrease of ventral tail tissues. (B) Wile type. (C) Rescued phenotype with ventral tail tissues appeared to be rescued. (D) Ventralized phenotype without head and axial structure like notochord. (E) Serious ventralized with disorganized structure. (F) The statistics analysis at 24 hpf for three injections (Amotl2 antisense MO, Amotl2 antisense MO+BMP RNA and BMPRNA alone) depends on different phenotypes. All the embryos anterior to the left and dorsal to the top. From the phenotype analysis showed that the defects of embryo patterning resulting from loss of amotl2 function can be at least partially rescued by the BMP signalling.

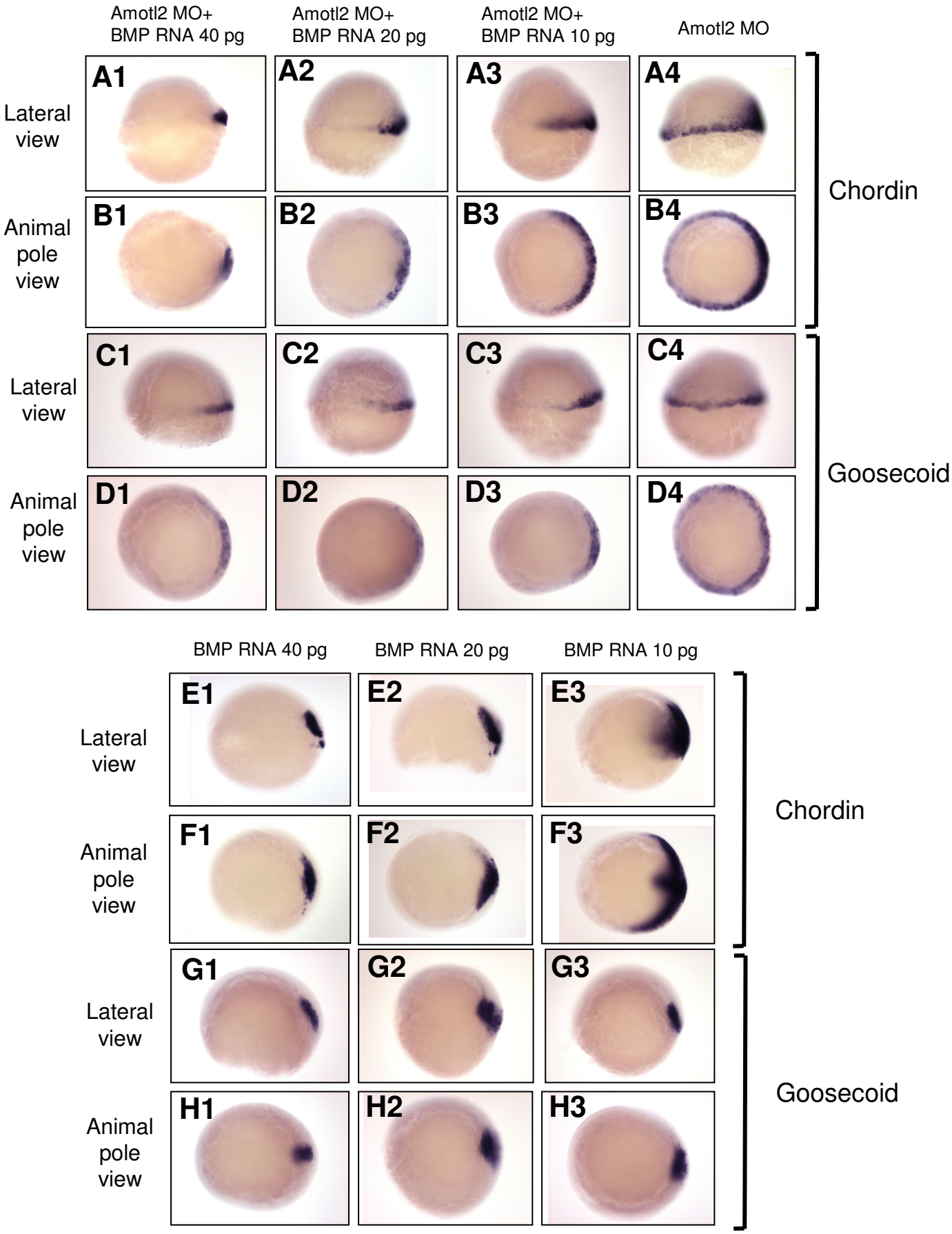


Figure II-15: The rescue of the Amotl2 knocked down embryo by the BMP overexpression checked by Chordin *in situ* hybridization at the shield stage. (A, E) Wild type, (B, F) Amotl2 antisense MO injected embryo. (C, G) Amotl2 antisense MO+BMP 20 ng RNA injected embryo. (D, H) BMP 20 ng RNA injected embryo. (A-D) Lateral view. (E-H) Animal pole view. Results showed that the marginal ventral expansion of chordin and goosecoid observed after amotl2 knock down can be gradually eliminated by increasing the amount of BMP RNA injected.

In addition, to determine the extent of this rescue, I probed the embryos by in situ hybridization using the dorsal markers chordin and goosecoid. Therefore, I injected different concentrations of BMP RNA (from 10 to 40 pg) with amotl2 antisense MO at the 1 cell stage then checked the expression of these markers at the shield stage. Results showed that the marginal ventral expansion of chordin and goosecoid observed after amotl2 knock down can be gradually eliminated by increasing the amount of BMP RNA injected (Fig. II-15A1-4, B1-4, C1-4, D1-4). The result above indicates that the dorsalization phenotype (both loss of ventral tissue and ventral expansion of dorsal markers) due to amotl2 lost of function is likely due to a decrease of BMP signalling because of ectopic expression of chordin in lateral and ventral margin.

D. Conclusion and Discussion

1. Relationships between Cdc42, Src, Ecdh1 and Amotl2.

After loss of function of amotl2, cells lose their migration ability. I show that this is not related to a defect in BMP signalling but is due to a decrease and disorganisation of actin filaments in amotl2 morphant cells (Huang et al., 2007). The round shape of these cells indicates that they have lost their polarity. The cell polarity is known to depend on Cdc42 GTPase activity, however, co-injection of active form Cdc42 RNA failed to rescue the amotl2 loss of function phenotype. This indicates that the methods I used to try a rescue the amotl2 impairment is not appropriate. Cdc42 is active toward the front of migrating cells (Itoh et al., 2002), and both inhibition and global activation of Cdc42 can disrupt the directionality of migration. In my rescue experiment, the active form Cdc42 is expressed in the whole cell and not on one side of the cell that would become the front of the migration. I would need to use another method that generates an asymetric repartition of activated Cdc42 inside the cell in order to properly address its ability to rescue the amotl2 loss of function phenotype.

In addition, the decrease of actin filaments formation after amotl2 inhibition may be the reason of the absence of formation of cell protrusions. Actin polymerization is considered as the main mechanism for the formation of lamellipodia. This happens close to the membrane (Pollard and Borisy, 2003). Formation of actin filaments requires series of steps including targeting the actin monomers to the protrusion end, branching the pre-existing filaments and regulating filaments length by capping the ends or promoting dissociation of actin from the end (Ridley et al., 2003). Many regulators are involved in these processes as profilin for actin monomer targeting (Pollard and Borisy, 2003),

ADF/cofilin for actin dissociation (Ridley et al., 2003), Arp2/3 complexes for filament branching, cortactin for filament branches stabilization and filamin A for the stabilization of the entire network (Welch and Mullins, 2002). Because the number of actin filaments is decreased and because they are disorganized in the amotl2 morphants, it may be worth to try these regulators of actin filament formation for future rescue experiments.

Rho GTPase including Rac, Cdc42 and RhoG are known to be required for lamellipodia and filopodia formation. Cdc42 is also required for cell polarity formation. Therefore, Rho GTPase family members play an important role both on cell polarization and protrusion formation. During the cell migration process, different GTPases, kinases and phosphatases distribute asymmetrically in the cell. For example, Cdc42 and the Rac GTPase will be activated by PI3Kinase at the leading edge promoting actin polymerization and the recruitment and clustering of integrin which promotes the adhesion of the cell (Ridley et al., 2003). In contrast, in the rear of cell the phosphatase PTEN will indirectly inactivate Cdc42 GTPase activity and Rho will inactivate the Rac activity (Devreotes and Janetopoulos, 2003; Merlot and Firtel, 2003), thereby preventing Rac-mediated protrusion at sites other than leading edge. All together, the asymmetry of the different molecular signals contribute to the cell polarity and formation of protrusions, therefore a rescue through a whole overexpression of one of these molecules is not likely to work. In the present study this failure to rescue amotl2 inhibition by the injection of an active form of Src indicates that although active form Src can be synthesized, its transportation from the perinuclear region to peripheral cell-matrix adhesion is defective. This may be due to the lost of amotl2 which may function as a carrier.

2. The possible reasons for which the migration defect cells change their patterning.

Study on the Src-related kinases, Yes, Known regulators of cytoskeleton, shows results similar to amotl2 phenotype (Tsai et al., 2005). Blocking yes expression by antisense MO shows the epiboly stopped at very early stages, before dome stage. When I inject the higher concentration of amotl2 antisense MO, the epiboly cannot occur and is blocked at the sphere stage. The patterning defects in amotl2 knock down situation is also similar to Yes (Tyrosine kinase) knock down phenotype (Tsai et al., 2005). In knock-down Yes embryos, early stage expression markers like goosecoid and bozozok/dharma decrease. Comparing to Yes inhibition, using high concentration of amotl2 antisense MO conditions, injected embryos fail to express genes normally activated at early stages like goosecoid and chordin (compare Fig. II-16 A, C and B, D; E, G and F, H). These data show that when inhibition of expression on molecules related to the Src-kinase pathway, either the translocation of Src-kinase (amotl2) or the kinase activity itself (yes), the epiboly cannot take place. In addition, these Src-kinase signalling impairment embryos show early patterning defects. As a result, the function of Src-kinase may contribute not only to cell migration but also to early patterning regulation.

My results show that zygotic Wnt8 transport in the cell may be impaired by amotl2 inhibition and suggest that amotl2 may not only respond to Src-kinase translocation but also for protein secretion. Since an early report shows that angiomotin but not angiomotin like 2 can indirectly activate Cdc42 through inhibition of Rich1 (Wells et al., 2006), it worth to discuss if changes in the cell migration will also impair the protein secretion. As a result, I tried to examine the role of GTPases on protein secretion. According to early reports, Cdc42 GTPase is responsible for vesicular transport specifically to the basolateral

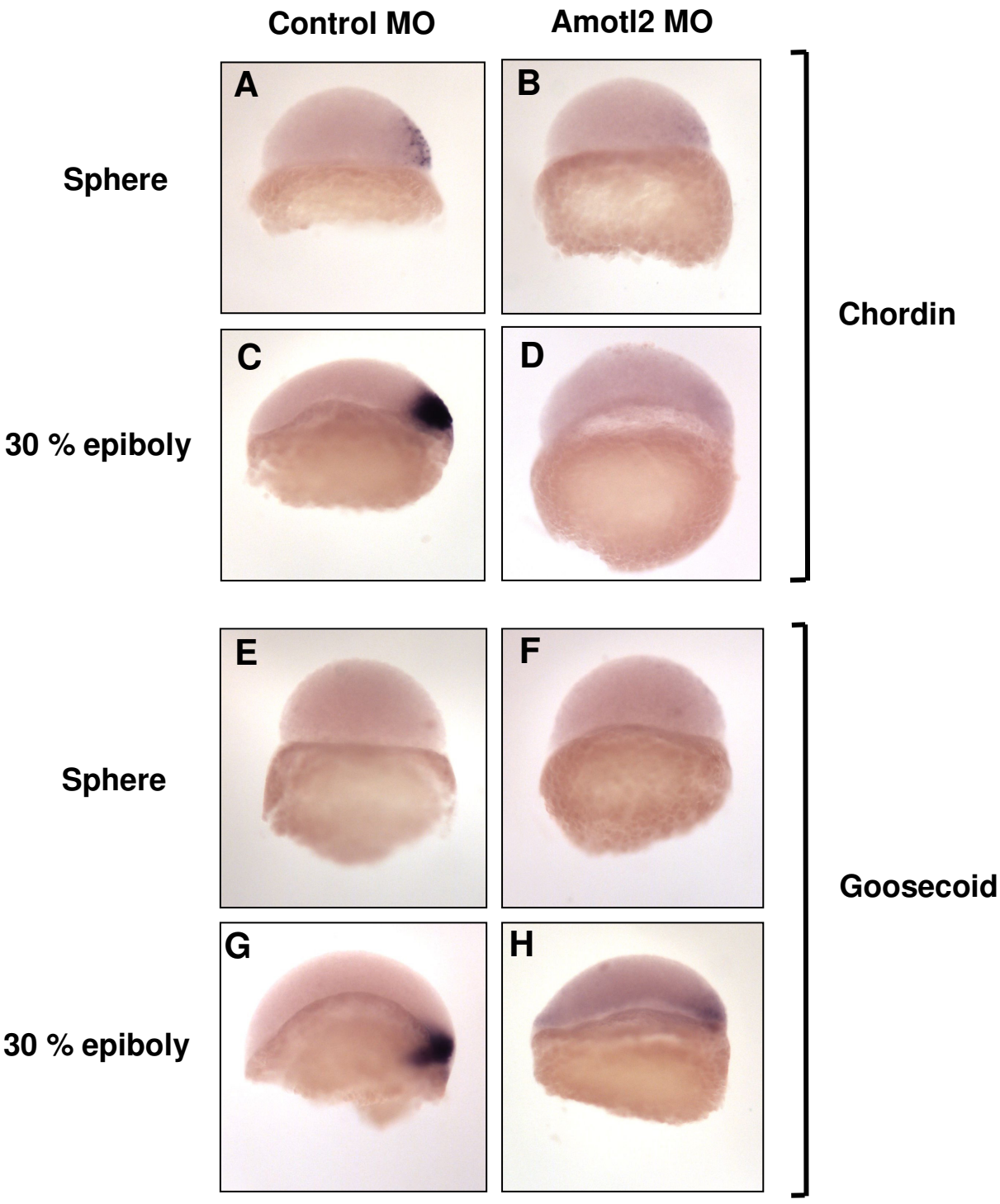


Figure II-16: The expression of Chordin and Goosecoid in the Amotl2 sense (Control) or antisense MO injected embryos. The embryos are injected with Amotl2 sense MO (Control, A, C, E, G) or Amotl2 antisense MO (B, D, F, H) at high concentration (800 nM) then collected at sphere (A, B, E, F) and 30%epiboly (C, D,G,H) stages. The collected embryos were staining with Chordin (A-D) or (E-H). All the embryo are lateral view and dorsal to the right. The result showed that high concentration amotl2 MO conditions injected embryos fail to express genes normally activated at early stages like goosecoid and chordin.

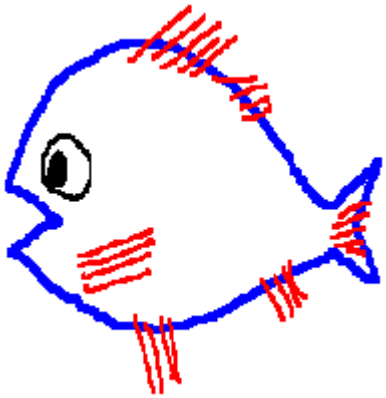
surface in MDCK (Madin-Darby Canine Kidney) mono cell layers leading to a selective depolarization of basolateral membrane proteins (Kroschewski et al., 1999). This vesicular transport is mediated by exocyst complex and Cdc42 GTPase interacts with its component Sec3p (Zhang et al., 2001). Similarly, Cdc42 can activate Ral, another small GTPase in the Ras family and interacts with another exocyst component, Sec5 (Moskalenko et al., 2002; Sugihara et al., 2002). These data suggest that Cdc42 may coordinate actin polymerization, vesicle docking machinery for polarized secretion. Compared to Cdc42 GTPase, amotl2 inhibition results in decreasing and disorganizing actin expression. This may explain why the secretion of Wnt8 may be affected. But further experiments are required to confirm this hypothesis. I will be particularly interested to see whether the actin polymerization structure and function in amotl2 knock-down embryos can restore Wnt8 secretion.

In addition to affect the protein transport, Cdc42 GTPase also contributes to transcriptional regulation of a TGF-β family member, Dpp (*decapentaplegic*) through regulation on the Jun N-terminal kinase (JNK) mitogen-activated protein (MAP) kinase (Stronach and Perrimon, 1999). As a result, inhibition of amotl2 may also regulate BMP (Dpp analog in zebrafish) transcriptional expression directly. But this hypothesis should be tested using BMP response element reporter (BRE) (Hata et al., 2000) to see the direct effect of amotl2 inhibition in vivo or in vitro.

Taking all data together, from my study I learnt that amotl2 is required for cell migration autonomously and may relate to actin polymerization mechanism which controls both cell polarity and cell protrusion. It is also required for non-receptor tyrosine kinase Src translocation to the terminal part of lamellipodia which is required for the migrating cell rear adhesion disassembly and retraction (Alahari et al., 2002; Webb et al., 2002). Loss of

function of amotl2 will change the whole pattern and result in increasing dorsal genes expansion accompanied by ventral genes reduction and may be linked to loss of ventral signals secretion. As a result, amotl2 plays an important role on zebrafish early patterning and cell movement control.

Part III.
The role of FrzA (SFRP1, Secreted Frizzled Related Protein 1) in early zebrafish development



A. Introduction

1. Isolation of FrzA during a genome wide in situ hybridization screen.

Knowledge of the spatiotemporal expression of a gene during embryogenesis provides valuable information about its potential contributions to the processes of development. The study of embryonic gene pattern profiles has been compared to DNA microarray technology (Niehrs and Pollet, 1999). Although DNA chips allow the simultaneous analysis of the transcription of a very large number of genes under a relatively restricted range of physiological conditions, patterns of gene expression at a various time point of embryogenesis also provide other valuable information on specific populations of cells or tissues.

The zebrafish is one of the most commonly used models which is suitable for large-scale in situ hybridization screening of the entire genome because it is easy to obtain a large number of eggs which are transparent. These advantages prompted us to perform a large-scale in situ hybridization screening of gene expressions during the embryonic development of zebrafish.

The stages of zebrafish embryo selected for the in situ hybridization study included the 75% epiboly, 3-somite, 14~20-somite, 24-, 36-, and 48-hpf stages. The results can be divided into three categories, those displaying spatially restricted expression, those with very widespread or ubiquitous expression, and those with no signal. The expression profiles we determined are all released to the zFIN website (http://zfin.org/cgi-bin/webdriver?MIval=aa-xpatselect.apg) for free searching.

FrzA (SFRP1) was isolated based on its unique expression pattern which is described in detail in the results section.

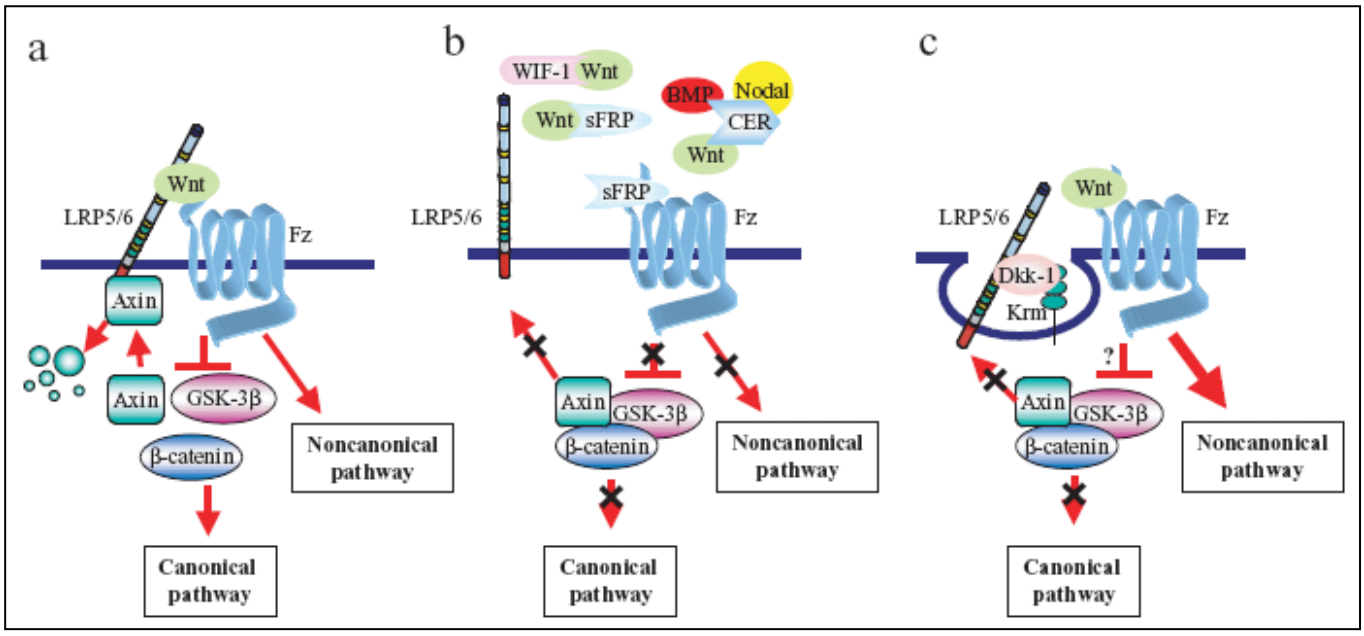


Figure III-1 : Regulation of Wnt signalling by antagonists. (Photograph from Kawano and Robert, 2003) (a) Activation of the canonical pathway is initiated when Wnt associates with Frizzled (Fzd) and LRP5/6. Subsequent events include the recruitment of Axin to LRP5/6 and its degradation, and the phosphorylation of dishevelled, resulting in disruption of the link between β-catenin and GSK-3β. β-catenin is no longer phosphorylated and is thus stabilised. Activation of the noncanonical pathway may involve interaction of Wnt with Fz in the absence of LRP5/6. (b) Antagonists such as sFRPs, Cerberus (CER) and WIF-1 prevent Wnt from binding to its receptors. In this case, both the canonical and the noncanonical pathways are inactivated. sFRPs may also inhibit Wnt by binding to Frizzled. (c) Dkk-1 interacts with LRP5/6 and the co-receptor Kremen 1/2 (Krm, green), and this triggers LRP5/6 endocytosis, thereby preventing formation of the LRP5/6–Wnt–Frizzled complex. Axin brings together the proteins that promote β-catenin phosphorylation, enabling b-catenin degradation and inhibition of the canonical pathway. The Wnt-Fzd complex can still activate the noncanonical pathway.

2. SFRP molecules.

SFRPs were originally found to be antagonists that bind directly to Wnts and inhibit Wnt signalling.

a. Classes of SFRPs

Wnt antagonists (table 1.) can be divided into two functional classes; the first is the SFRP class which includes the SFRP family, WIF-1, and Cerberus, that bind directly to Wnts, thereby altering their ability to bind to Wnt receptor complexes (Fig. III-1b). The second is the Dickkopf class which comprises certain Dickkopf family proteins, which inhibit Wnt signalling by binding to the LRP5/LRP6 component of Wnt receptor complexes (Fig. III-1c) (Kawano and Kypta, 2003).

There are eight members in the SFRP family: SFRP1~5 (SFRP3 is better known as FrzB), Sizzled, Sizzled 2, and Crescent. On the basis of sequence homology, SFRP1, SFRP2, and SFRP5 form a subgroup; SFRP3 and SFRP4 form another subgroup; and Sizzled, Sizzled2, and Crescent form a third group. The function of Sizzled as a Wnt signalling inhibitor was previously controversy (Bradley et al., 2000; Collavin and Kirschner, 2003; Salic et al., 1997). It is currently known to bind to Xolloid and to prevent Chordin cleavage by Xolloid which functions in the BMP signalling pathway and not in the Wnt pathway (Lee et al., 2006). Same conclusion also been shown in the zebrafish which sizzled represses the activities of Tolloid-family proteins include Bmp1a and Tolloid-like 1(Tll1) by preventing them cleavage chordin. Loss of both Bmp1a and Tll1 function leads to a complete suppression and reversal of the sizzled mutant (*Ogon*) phenotype (Muraoka et al., 2006).

FrzB and Cerberus were discovered at the same time from a screening of *Xenopus* dorsal-specific factors (Bouwmeester et al., 1996). Most work on

Table 1. Wnt antagonist family molecules (table from Kawano and Robert, 2003)

Name(s)	Mechanism of action	Wnt antagonist activity	Wnt agonist activity/inhibition of antagonist activity
sFRP1 (FRP, SARP2, FrzA) SFRP1	Binds Wnt and Fz	Yes (Finch et al., 1997; Xu et al., 1998; Uren et al., 2000)	Agonist at low concentrations (Uren et al., 2000)
sFRP2 (SARP1) SFRP2	Binds Wnt	Yes (Ladher et al., 2000)	Inhibits sFRP1 (Yoshino et al., 2001)
sFRP3 (FrzB, Fritz) FRZB	Binds Wnt	Yes (Leyns et al., 1997; Wang et al., 1997a; Wang et al., 1997b; Lin et al., 1997; Mayr et al., 1997)	
sFRP4 (FrzB-2) SFRP4	Binds Wnt	?	
sFRP5 (SARP3) SFRP5	Binds Wnt	?	
Sizzled*	Binds Wnt (?)	Yes (Salic et al., 1997) No (Bradley et al., 2000; Collavin and Kirschner, 2003)	
Sizzled2*	Binds Wnt (?)	No (Bradley et al., 2000)	
Crescent*	Binds Wnt	Yes (Pera and De Robertis, 2000)	
WIF-1	Binds Wnt	Yes (Hsieh et al., 1999)	
Cerberus*	Binds Wnt	Yes (Piccolo et al., 1999)	
Coco*	Binds Wnt (?)	Yes (Bell et al., 2003)	
Dkk-1 (DKK1)	Binds LRP5/6	Yes (Glinka et al., 1998; Krupnik et al., 1999; Fedi et al., 1999; Wu et al., 2000; Brott and Sokol, 2002)	Inhibits Dkk-2 (Wu et al., 2000)
Dkk-2 (DKK2)	Binds LRP5/6	No (in *Xenopus* embryos, Krupnik et al., 1999; Wu et al., 2000) Yes (in *Xenopus* embryos, Brott and Sokol, 2002) Yes (in cell lines, Wu et al., 2000; Li et al., 2002)	Agonist (Wu et al., 2000) Agonist with LRP6 (Li et al., 2002; Brott and Sokol, 2002)
Dkk-3 (REIC) (DKK3)		No (Krupnik et al., 1999; Mao and Niehrs, 2003)	
Dkk-4 (DKK4)	Binds LRP5/6 (?)	Yes (Krupnik et al., 1999; Mao and Niehrs, 2003)	
Soggy (DKKL2)		No (Krupnik et al., 1999)	

Approved gene symbols are underlined.
*To date, mammalian genes for Sizzled, Sizzled2 and Crescent have not been identified. The mammalian gene related to Cerberus (mCer1) does not encode a Wnt antagonist, and the antagonist activity of mammalian Coco has not been tested.

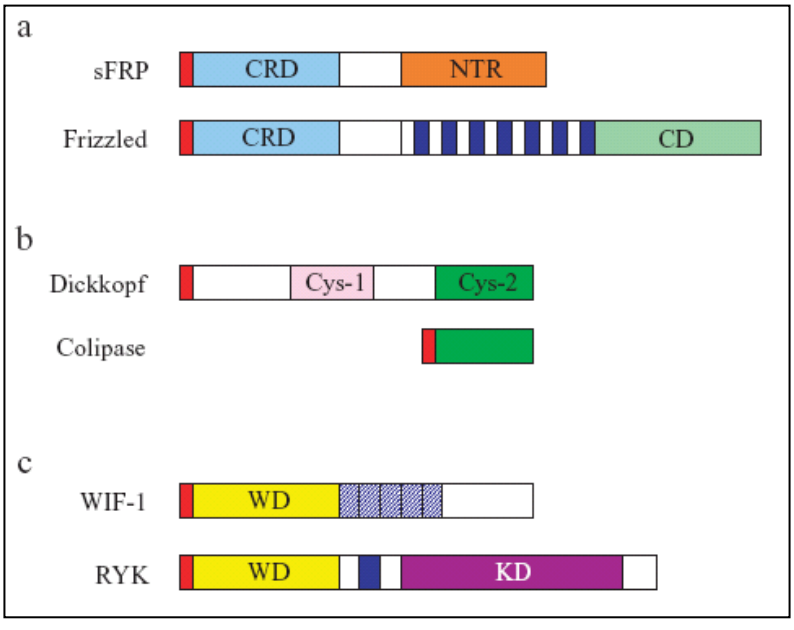


Figure III-2 : Wnt antagonists and related proteins. (Photograph from Kawano and Robert, 2003) (a) sFRP family proteins are related to Frizzled receptors in the CRD (Cysteine-rich domain). NTR, netrin-like domain; CD, cytoplasmic domain. (b) Dickkopf family proteins are related to pancreatic colipase in Cys-2 (Cysteine-rich domain-2). The domain containing 10 conserved cysteine residues in colipase is shown in green. Cys-1, Cysteinerich domain-1. (c) WIF-1 is related to the receptor tyrosine kinase, RYK in the WIF domain (WD). KD, tyrosine kinase domain. Signal peptides, transmembrane domains and EGF-like repeats are shown as red, blue, and hashed boxes, respectively.

FrzB in vertebrates has been on *Xenopus*, It is expressed in the spemann organizer during early gastrulation in a complementary pattern to Xwnt8. It can bind to XWnt8, and inhibits ectopic Xwnt8 function (Leyns et al., 1997; Wang et al., 1997a; Wang et al., 1997b).

b. The structure of SFRPs

SFRPs contain a cystine-rich domain (CRD) on their N-terminal half which shares 30%~50% sequence similarity with Frizzled proteins (Fig III-2a, (Melkonyan et al., 1997)). It is still not clear whether SFRPs interact with Wnt ligands through the CRD domain or the C-terminal domain (Lin et al., 1997; Uren et al., 2000). Many conflicting data from SFRP interactions with Wnts may be due to differential affinities among SFRPs and their different Wnt partners (Kawano and Kypta, 2003). In addition, the CRD of SFRP1 also appears to interact with itself and with Frizzled (Bafico et al., 1999). Thus, SFRPs may block Wnt signalling either by interacting with Wnt proteins to prevent them from binding to Frizzled proteins or by forming non-functional complexes with Frizzled.

The C-terminal half of SFRPs contains a domain that shares weak sequence similarity with the axon guidance protein, netrin (NTR, Fig III-2a). The NTR domain contains six cysteine residues and can also be found in tissue inhibitors like metalloproteases (Banyai and Patthy, 1999). Although SFRPs are secreted, several reports have shown that they are mainly found on plasma membranes and/or in the ECM in cell culture systems (Kawano and Kypta, 2003). In common with some Wnts, SFRP1 is released into the culture medium after treatment with heparin (Finch et al., 1997). However, another report shows conflicting result as SFRP1 is present on the extracellular matrix but not in the conditional medium (Bafico et al., 1999). As a result, no

conclusion is made on the secretion mechanism of SFRP1. In addition, the association between SFRPs and heparin sulfate proteoglycans is thought to stabilize SFRP-Wnt complexes or to determine antagonist localization (Uren et al., 2000).

c. The function of SFRP1

SFRP1 has been most intensively studied in cancer research, and it is downregulated in several cancers including cervical, breast, ovary, and kidney carcinomas (Ko et al., 2002; Ugolini et al., 2001; Zhou et al., 1998). Hypermethylation of the SFRP1 promoter (as well as those of SFRP2, SFRP4, and SFRP5) results in the downregulation of its expression which occurs at a high frequency in primary colorectal carcinomas (Suzuki et al., 2002). Consistent with its role in inhibiting cell proliferation, SFRP1 can promote greater sensitivity of MCF-7 breast cancer cells to TNF-induced apoptosis (Melkonyan et al., 1997). Bovine SFRP1 (called FrzA) is expressed during the formation of neovessels and becomes undetectable when the vasculature is fully mature. It also inhibits the growth of endothelial cells (Duplaa et al., 1999). As a result, data from SFRP1 studies show the inhibition of cell proliferation.

There are some reports concerning the role of SFRP1 in early vertebrate development. SFRP1 is expressed in zebrafish embryos including the forebrain (Duplaa et al., 1999; Tendeng and Houart, 2006), is required for its development, and functions as an antagonist of Wnt signalling (Lamy et al., 2006; Peng and Westerfield, 2006). It is also required for eye field formation in the zebrafish and Medaka fish (Esteve et al., 2004; Kim et al., 2007). SFRP1 is expressed in the early stages of *Xenopus* embryos and can regulate cell movement. SFRP1 is able to antagonize formation of secondary axes induced by overexpression of xWnt8 (Xu et al., 1998). In the mouse, SFRP1 and

SFRP2 can regulate anteroposterior axis formation and somite segmentation during embryogenesis (Satoh et al., 2006). All data for SFRP1 in early vertebrate development suggest that it functions as a Wnt signalling antagonist. But the mechanism is unknown.

The mechanism by which SFRP1 inhibits Wnts signalling has been widely investigated. Using a tissue culture system, a low concentration of SFRP1 can potentiate *Drosophila* Wg activity while a high SFRP1 concentration inhibits it (Uren et al., 2000). It suggests SFRP1 has high-affinity and low-affinity Wg binding sites; binding to high-affinity sites promotes Wg signalling, whereas binding to low-affinity sites inhibits it. However, this hypothesis has still not been proven. In addition, *Drosophila* Wg used in that experiment is not a vertebrate Wnt. Although they have a high degree of conservation, the interaction between SFRP1 and Wg still cannot represent SFRP1-Wnts interaction in vertebrates. Evidence of the direct interaction between SFRP1 and Wnts is only available for SFRP1 and Wg (from *Drosophila*) (Uren et al., 2000; Xu et al., 1998), mouse Wnt1 and Wnt2, or human Wnt1 (Bafico et al., 1999; Dennis et al., 1999). However, interactive data of SFRP1 with other Wnts are lacking. Nevertheless, there is another explanation for the inhibition mechanism of SFRP1 on Wnts signalling: SFRP1 can bind directly to the Frizzled receptor and form a non-functional complex, thus preventing Wnt binding (Bafico et al., 1999). Another mechanism has been also proposed in the chick retinal ganglion cell (RGC) growth as chick SFRP1 directs retinal ganglion cells (RGC) movement by activating the Frizzled 2 receptor but has been shown to be independent of Wnt5a and Wnt 7b (Rodriguez et al., 2005).

However, the exact contribution of SFRP1 to early vertebrate development is unknown. As well, the mechanism by which it inhibits Wnt signalling is still unclear. Therefore I used the zebrafish model to investigate the mechanisms

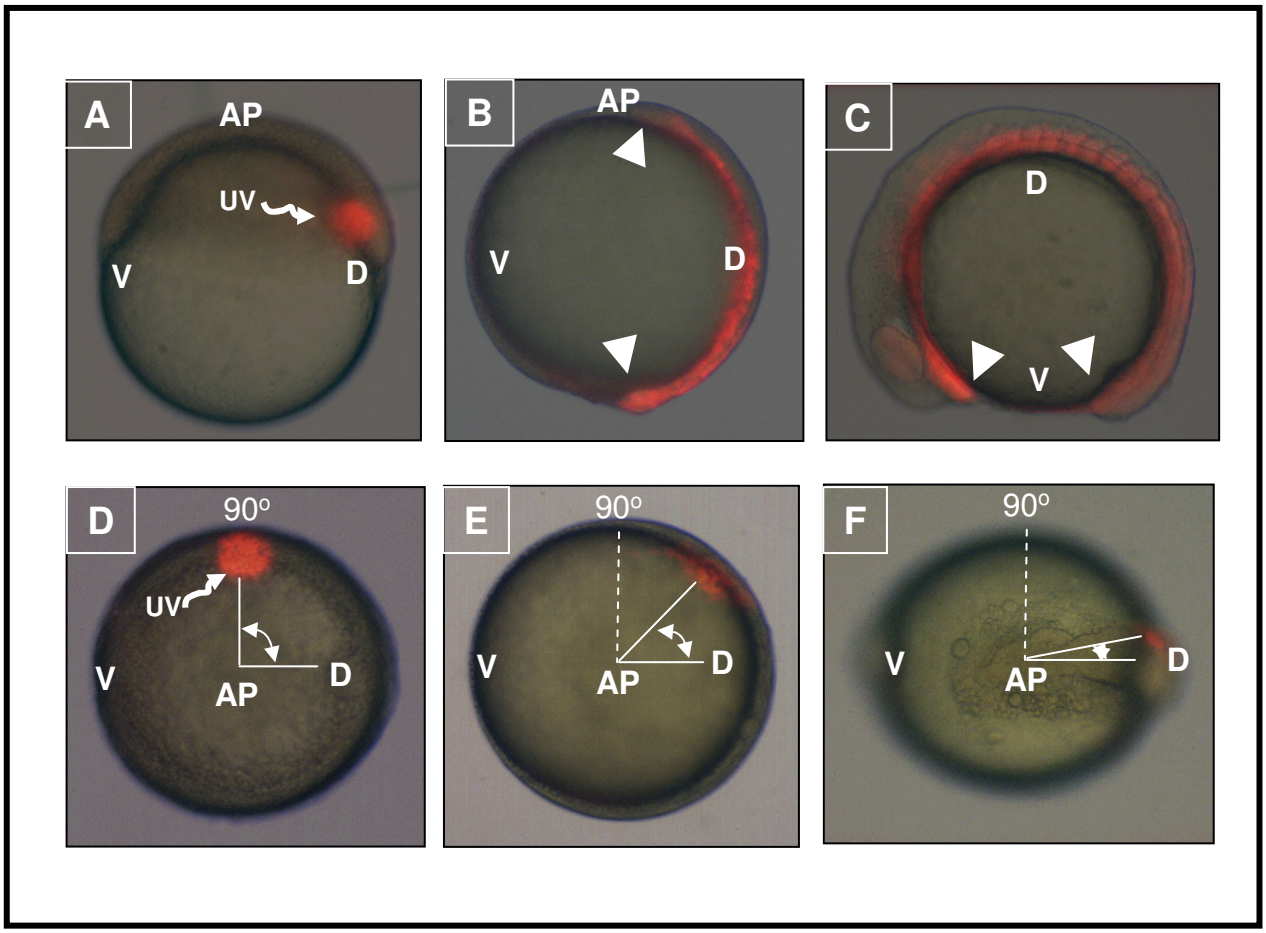


Figure III-3 : Measurement of Convergence and Extension cell movement on the zebrafish embryo. The Extension (A-C) movements are measured by the length of the labeled cells in red on the axis at the different developmental stages. The arrow head indicates the begin and the end of the labeled cells. The Convergence (D-F) movements are compared by the angle between of the labeled cells on the margin and the dorsal side at the different stages. The stages (A, D: shield, B, E: tail bud, C, F: somitogenesis). The abbreviations: D: Dorsal part, V: Ventral part, AP: Animal Pole. The extension (A-C) movements are lateral view and the Convergence (D-F) movements are animal pole view.

by which the zebrafish SFRP1 (FrzA) affects the early development of vertebrate embryo.

B. Specific experimental procedures

The measurement of the zebrafish embryo Convergence and Extension speed was according to the methods that described in the earlier papers (Myers et al., 2002b). We injected the Kaede GFP RNA (Ando et al., 2002) into the embryo at one cell stage. After the embryo growth until shield stage which can distinguish the dorsal and the ventral side. We use the microscope with the pinhole accessory to let the UV light (Microphot EPI-FL3, Nikon, Japan, 510 M filter) to specifically photo convert a group of cells either on the lateral or the dorsal margin from green into red. After photo conversion of the green Kaede to red form by the UV light for 30 seconds, we took a picture then transfer the embryo into the 2% cellulose with 7 cover slides bridge viewing chambers as protocol in the zebrafish book then keep it in the humid box at 28 degree until the next sampling time. After two and five hours, we take the picture with the same magnification as the original then measure the extension length or the convergence angle by the ImageJ software (Fig III-3A-F).

C. Results

1. Structure and expression pattern of FrzA (SFRP1) in the zebrafish embryo

a. Analysis of the structure of the zebrafish FrzA

Within the course of a large scale *in situ* hybridization screen intended to characterize genes whose expression is spatially and temporally restricted during embryonic development (Thisse et al., 2001a), we isolated a cDNA

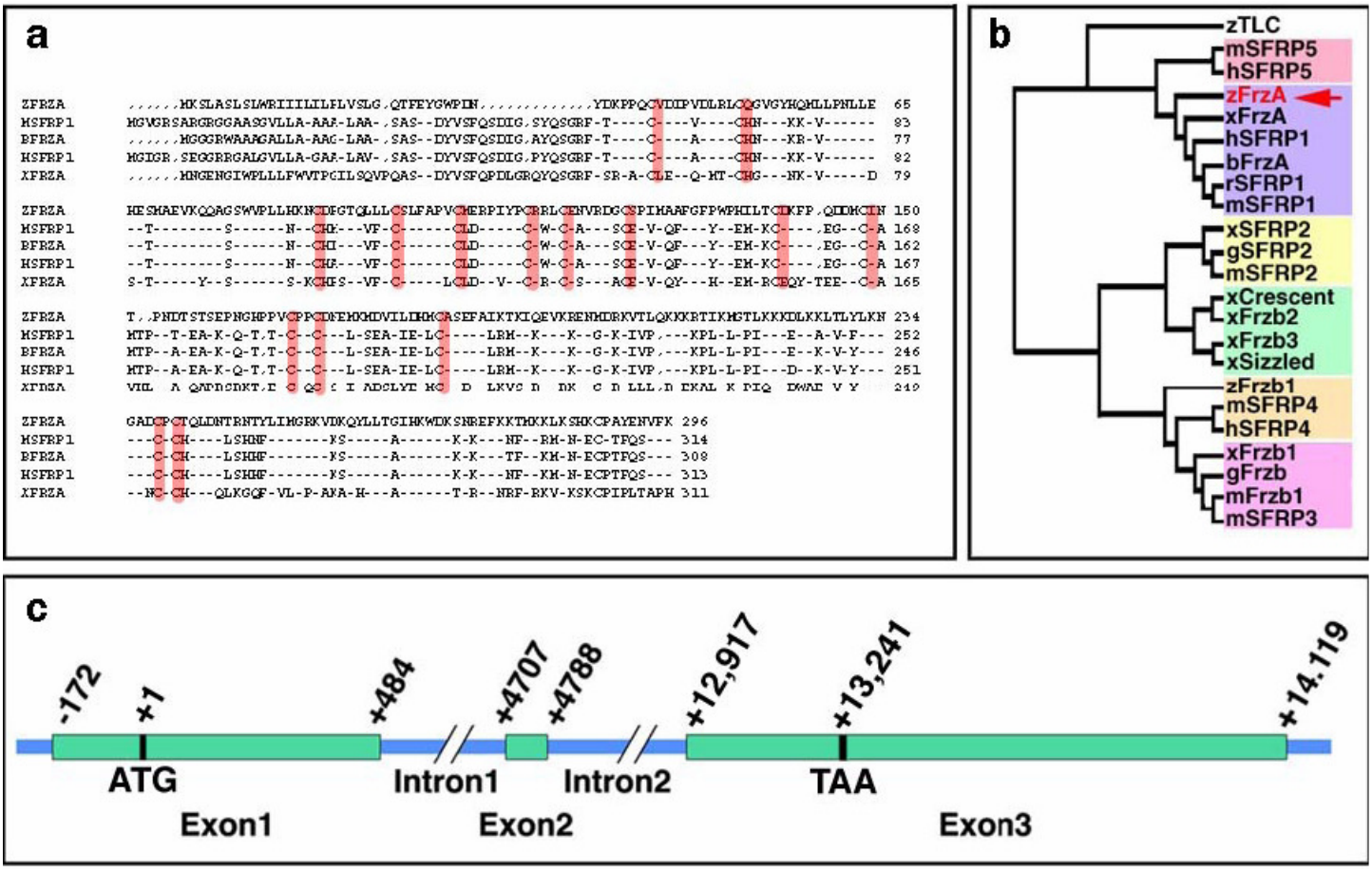


Figure III-4: Identification of the zebrafish FrzA gene and structural relationships with other members of the SFRP family. (a) Amino-acids sequence alignment of the vertebrate members of the SFRP1 class of proteins. Dashes indicate identical or similar amino acids; Dots indicate gaps that have been introduced to optimize the alignment. Cysteine residues characteristic of the CRD domain of the SFRP family are in red. Gene accession numbers (GenBank database): MSFRP1 : |AAC53145 , BFRZA : NP_776885, HSFRP1 : NP_003003 , XFRZA : AAD02492 , FRZA : AY307179 (b) Philogenetic tree illustrating the relationship of FrzA with other members of the SFRP family. zFrzA belongs to the subgroup of SFRP1/FrzA. (c) Intron-exon organization of zFrzA transcription unit. Nucleotides are numbered on top with +1 corresponding to the ATG codon for translation initiation.

fragment sharing homologies with SFRPs (Rattner et al., 1997). The full-length coding sequence of the corresponding gene has been isolated by screening a gastrula stage cDNA library. Subsequent sequencing of this 1939bp cDNA allowed the identification of a 888 nucleotide long open reading frame encoding a 296 amino-acids protein sharing 65.7% identity with bFrzA/SFRP1 and 54.1% with xFrzA/SFRP1 (Fig. III-4). We observe a strong conservation of the CRD (cystein rich domain) characteristic of the Wnt interacting domain on Frizzled receptors. The phylogenetic tree of the SFRP family revealed that the identified gene belongs to the SFRP1/FrzA subclass and corresponds to the zebrafish FrzA (Fig. III-4b). The sequence of FrzA transcription unit has been identified after a blast search against the zebrafish genome database (http://pre.ensembl.org/Danio_rerio/) on contig ctg11796 and the genomic organization of the zFrzA locus is shown in Fig. III-4c. The FrzA gene presents three exons (656 pb, 81pb and 1202 pb) and two introns (4223 pb and 8129 pb).

b. Expression pattern of FrzA

(1). Temporal and spatial expression

Temporal and spatial expression pattern of FrzA has been analyzed on whole mount *in situ* hybridization. This gene displays a very complex and dynamic expression pattern during embryogenesis, in various structures and organs, suggesting multiple developmental roles during embryonic development.

(2). FrzA blastula and gastrula expression

Analysis performed at the fertilized and 1 cell stage embryo by RT-PCR showed that FrzA is maternally expressed (Fig. III-5) although it is not detected

by whole mount *in situ* hybridization. By in situ hybridization, FrzA transcripts are first detected at the sphere stage soon after the initiation of the zygotic genome transcription. Expression, initially ubiquitous (not shown), becomes spatially restricted at 30% epiboly (5hpf). Staining is observed in the animal part of the blastula embryo as well as in a subset of cells lying at the margin (Fig. III-6a, b). At the beginning of gastrulation, marginal cells expressing FrzA involute under the epiblast all around the margin (Fig. III-6c, d, e). Ectodermal expression in the animal pole part of the gastrula becomes stronger in the presumptive anterior brain territory (Fig. III-6c). We did not detect any expression in extraembryonic structures such as the enveloping layer (Fig. III-6f) or the yolk syncytial layer (not shown). At 75% epiboly, FrzA is expressed in hypoblast, except in the chorda-mesoderm. A labeling is also observed in forerunner cells (Fig. III-6g - l). In epiblast, expression is restricted to presumptive anterior neural plate (Fig. III-6h, i, l). At the end of gastrulation, FrzA transcripts are detected in the hypoblast and in Kupffer's vesicle (Fig. III-6m - q). Hypoblastic expression is stronger in the presumptive segmental plate and weaker in head mesoderm (Fig. III-6 n). Expression strongly increases in the anterior neural plate, except in the axis (Fig. III-6n).

(3). Expression during somitogenesis

At 12hpf (5 somite stage) FrzA is strongly expressed in the brain: telencephalon, eye-field and presumptive anterior rhombencephalon (Fig. III-7a-d). Double labeling with Krox20 (which labels rhombomeres 3 and 5 - (Oxtoby and Jowett, 1993)) confirms that transcripts are located in the hindbrain anterior to rhombomere 3 (Fig. III-7d) where FrzA RNAs are excluded from the dorsal most part (Fig. III-7c). In the trunk, transcripts are detected in unsegmented paraxial mesoderm and in nascent somites in which

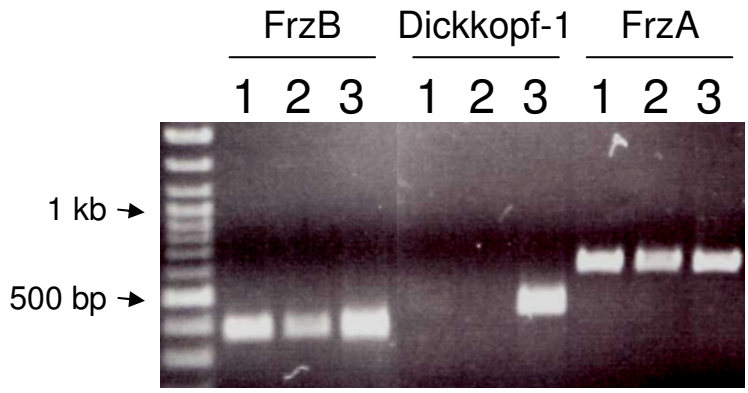


Figure III-5: The expression of different Wnt pathway inhibitors (FrzB, Dickkopf-1, FrzA) detected by RT-PCR: Numbers indicate different developmental stages. Fertilized embryo(1), 1 cell stage(2), 90% epiboly(3). The RT-PCR result indicates FrzA as FrzB is maternally expressed.

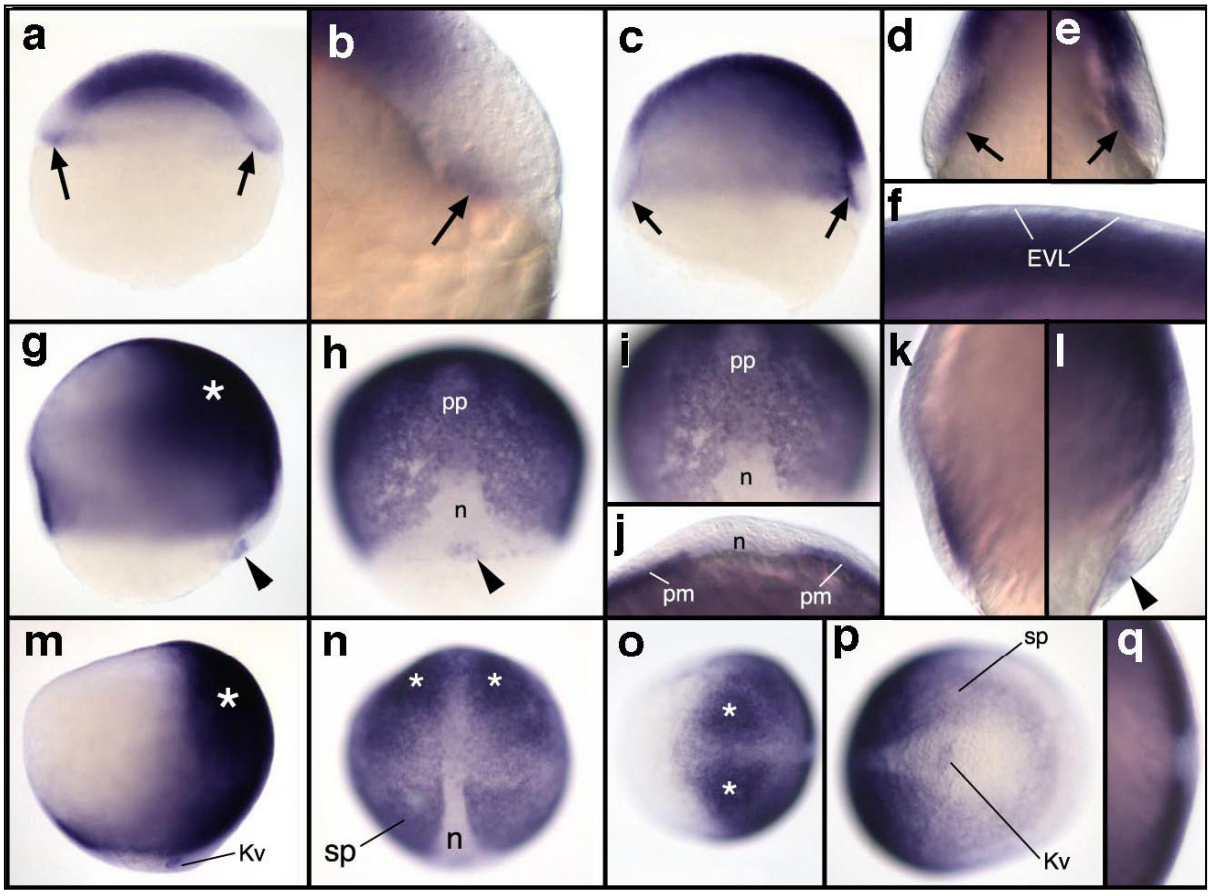


Figure III-6: Expression pattern of FrzA at blastula and gastrula stages
(a) Side view of an embryo at 30% epiboly. Arrows marginal expressing cells. (b) High magnification of the margin of the embryo in (a). (c) Side view of an embryo at shield stage. Arrows indicate the internalized hypoblast. (d-e) High magnifications of (d) ventral and (e) dorsal marginal zones of the embryo in (c). (f) High magnification of the animal pole of the embryo in (c). (g) Side view of an embryo at 75% epiboly. Star indicates anterior neural plate and arrowhead the forerunner cells. (h - i) Dorsal view of the embryo in (g). zFrzA is expressed in the prechordal plate (pp) but absent from the presumptive notochord (n). Arrow : forerunner cells. (j) Optical cross-section confirming the presence of transcripts in paraxial mesoderm (pm) and their absence in posterior neural plate and presumptive notochord (n). (k - l) High magnification of (k) ventral and (l) dorsal marginal zones of the embryo in (g). Arrowhead in (l) : forerunner cells. (m) Side view of an embryo at the end of gastrulation showing strong expression in anterior neural plate (star), posterior mesoderm and Kupffer's vesicle (Kv). (n) Dorsal view of the embryo in (m). Anterior neural plate (stars), sp : segmental plate, n : notochord. (o) Dorsal view of the anterior neural plate (stars) of the embryo in (m). (p) Dorsal view of the posterior part of the embryo in (m). (q) Optical cross-section at the level of the segmental plate showing the absence of staining in the notochord. Embryos are oriented animal pole up and dorsal to the right except (o, p) anterior to the left.

they progressively disappear from the posterior part (Fig. III-7e). At the 14 somite stage, the expression in the rhombencephalon extends and covers rhombomeres number 1, 2, 3 and 5 (Fig. III-7f, h) as confirmed by double labeling with Krox20 (Fig. III-7i). Labeled cells are confined to the ventral part of the hindbrain (Fig. III-7f, j). A weak staining is also visible in ventral midbrain (Fig. III-7f). FrzA is not detected in the tail bud region whereas it is expressed in the posterior spinal cord (Fig. III-7k). More anteriorly, its expression is restricted to ventral and dorsal neurons (Fig. III-7f, n) with a stronger staining for the ventral domain (Fig. III-7m). In mesoderm, somites still express FrzA (Fig. III-7g, k). At the 24 somite stage (21 hpf) brain expression is still confined to the forebrain and eye, posterior midbrain (presumptive tegmentum) and hindbrain. The anterior midbrain as well as the midbrain-hindbrain boundary is unlabeled (Fig. III-7q, r). The ventral diencephalon (Fig. III-7v) and the olfactory placodes also accumulate FrzA transcripts (Fig. III-7u, v). In the rhombencephalon, the expression is restricted to ventral position (Fig. III-7p, t) with a strong staining in the presumptive cerebellum (Fig. III-7p - s). Some cells located in a more dorsal position are labeled in rhombomeres 4, 5 and 6 (Fig. III-7t). Posteriorly (Fig. III-7p), the neural expression is now restricted to the ventral spinal cord, posterior most somites and presumptive gut (Fig. III-7w, x). A patch of expression is noted in ventral mesodermal cells near the tail bud (Fig. III-7y).

(4). Expression during late embryogenesis

At 24hpf FrzA is still expressed in the central nervous system (Fig. III-8a). From anterior to posterior transcripts are observed in forebrain ventricular zone, in ventral diencephalon, midbrain (tegmentum) (Fig. III-8a, b), in ventral hindbrain (Fig. III-8a, c) with a weaker staining extending in more dorsal

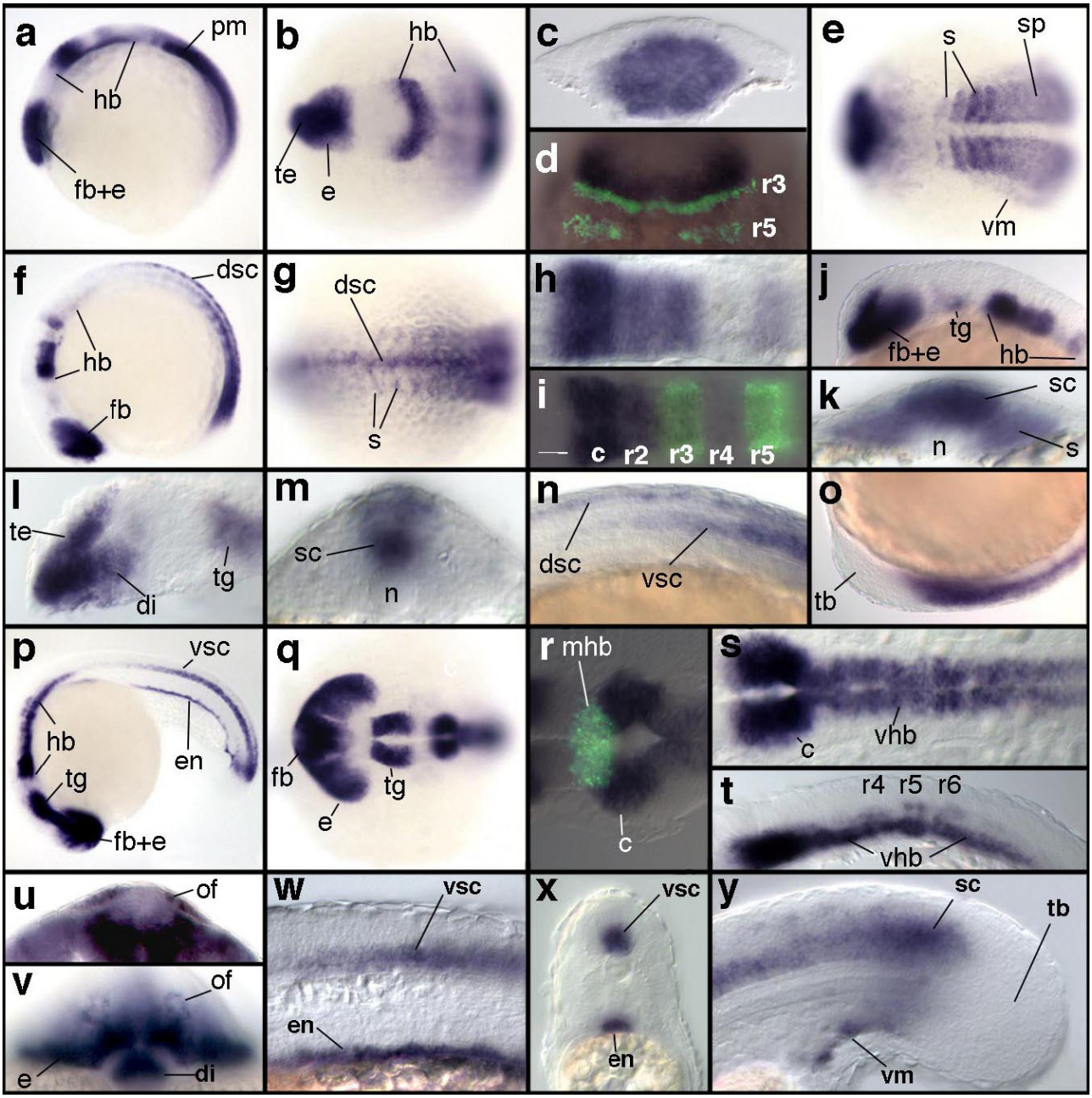


Figure III-7: Expression pattern of FrzA during somitogenesis

Embryo at the 5 somite stage (a) side view (b) dorsal view of head region (e) dorsal view of trunk region. (c) thick section at the level of the presumptive hindbrain (d) dorsal view of the hindbrain region labeled with Krox20 (green) and FrzA (blue). Embryo at the 12 somites stage (f) lateral view, (g) dorsal view of the trunk, (h) dorsal view of the hindbrain, (i) double labeling with Krox20 (green), (j) side view of the head region, (k) thick section in the posterior region (l) high magnification of the forebrain in lateral view, (m) thick section in the trunk, (n) high magnification of the trunk region in lateral view (o) high magnification of the tail bud region in lateral view.

Embryo at the 24 somites stage: (p) side view (q) dorsal view of the head (r) double labeling with en2 (green) which identifies the midbrain hindbrain boundary (mhb), (s) dorsal view and (t) lateral view of the hindbrain, (u) transverse optical cross section at the level of the olfactory placode, (v) frontal view, (w) high magnification of the trunk region (x) thick section at the level of the trunk, (y) high magnification of the tail bud region. c: cerebellum, di: diencephalon, dsc: dorsal spinal cord, en: endoderm, fb+e: forebrain and eye, hb:hindbrain, n: notochord, of: olfactory placode, r2-r5: rhombomeres 2-5, s: somites, sc: spinal cord, sp: segmental plate, tb: tail bud, te: telencephalon, tg: tegmentum, vhb: ventral hindbrain vm: ventral mesoderm, vsc: ventral spinal cord.

position (Fig. III-8a), in the ventral part of the posterior spinal cord (Fig. III-8a, k, l) as well as in the dorsal part of the caudal most neural tube (Fig. III-8a, l). FrzA transcripts are also detected in sensory organs. A staining is observed in the olfactory vesicle (Fig. III-8b), in the ventral part of the otic vesicle (Fig. III-8c, g), and in the anterior and posterior lateral line (Fig. III-8a, c-f, h) including the lateral line primordium (Fig. III-8e, f). In mesoderm, FrzA RNAs accumulate at the ventral most part of the tip of the tail, and in the corpuscle of Stannius (Fig. III-8a, l, j). Finally FrzA is also expressed in endodermal derivatives, in liver and pancreas primordia (Fig. III-8d, h) and, in the trunk part, in the presumptive gut (Fig. III-8a, i). One day later, FrzA expression has strongly decreased from the central nervous system and is only maintained in tegmentum and at the tip of the spinal cord (Fig. III-8p). It is still detected in lateral line (anterior – Fig. III-8m, q, u, posterior – Fig. III-8m - o, q and dorsal – Fig. III-8m, q - s) as well as in ventral part of the otic vesicle (Fig. III-8t). The corpuscles of Stannius are still labelled (Fig. III-8m) and a strong expression is now observed in splanchnocranium and pectoral fin cartilage (Fig. III-8m, q, u).

2. Effect of gain and loss of function of FrzA

a. FrzA regulates the zebrafish Dorsoventral and Anterior-posterior patterning

I investigated the function of FrzA through loss of function experiments by injection of antisense MO or by gain of function through injection of sense FrzA RNA.

(1) Morphological analysis

Analysis of FrzA loss-of-function

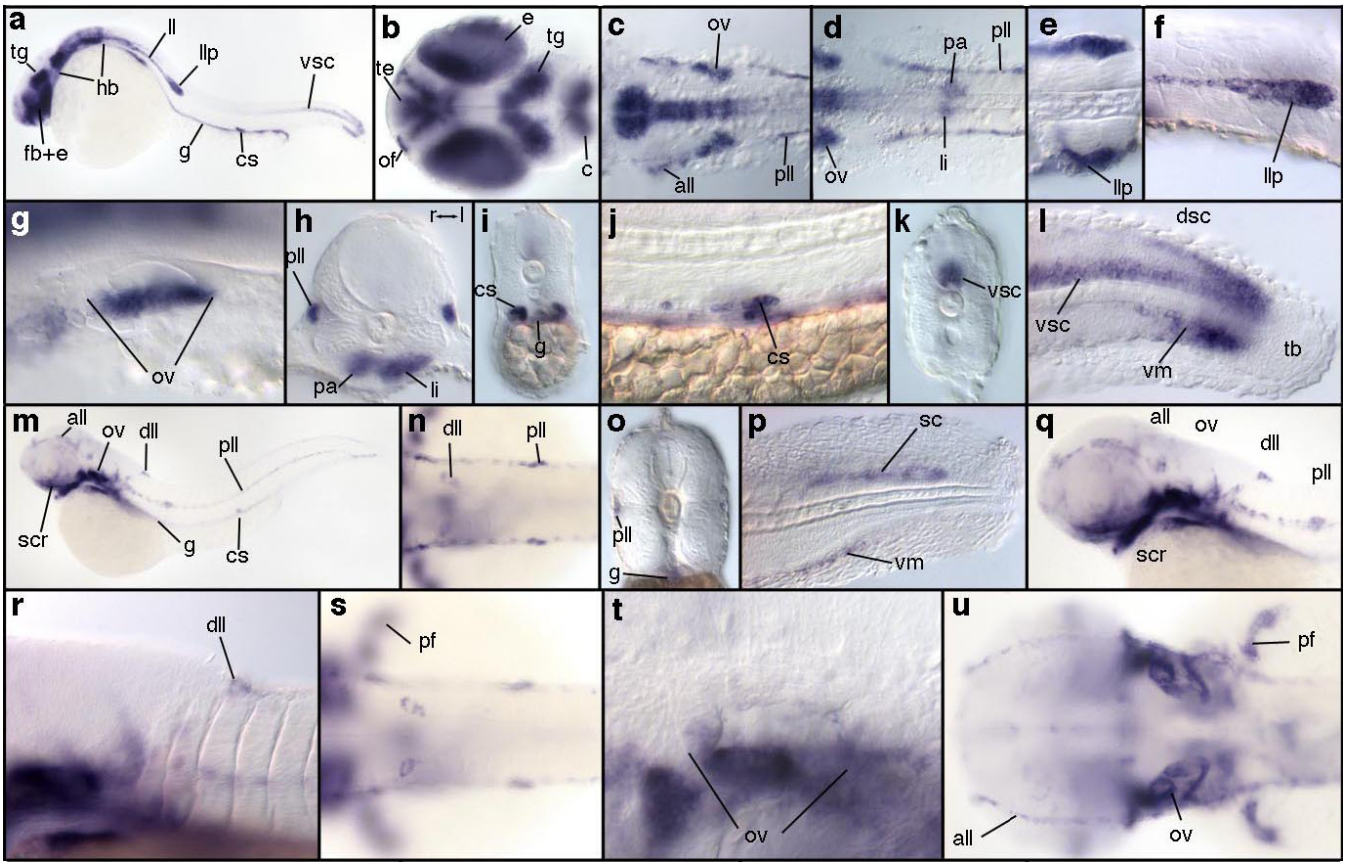


Figure III-8: Expression pattern of FrzA from pharyngula period to hatching
Embryo at prim5 (24 hpf): (a) side view, (b) dorsal view of the head, (c) dorsal view of hindbrain, (d) dorsal view of anterior trunk, (e-f)) high magnification showing the posterior lateral line primordia (llp) (e) dorsal view, (f) lateral view. (g) lateral view of the otic vesicle (ov), (h) thick section at the level of liver (li) and pancreas (pa) primordia, (i) thick section at the level of the corpuscle of Stannius (cs), (j) lateral view of the posterior trunk, (k) thick section of the tail, (l) lateral view of the tail.
Embryo at long-pec stage (48 hpf): (m) side view (n) dorsal view of the trunk (o) thick section at the trunk level (p) lateral view of the tail (q) lateral view of the head (r) lateral view of the anterior trunk (s) dorsal view of truncal region, (t) lateral view of the otic vesicle, (u) dorsal view of the head. all: anterior lateral line, c: cerebellum, dll: dorsal lateral line, dsc: dorsal spinal cord, fb+e: forebrain and eye, g: gut, hb:hindbrain, of: olfactory placode, pf: pectoral fin, pll: posterior lateral line sc: spinal cord, scr: splanchnocranium, tb: tail bud, tg: tegmentum, vm: ventral mesoderm, vsc: ventral spinal cord.

In order to assess the role of FrzA during embryogenesis I inactivated the function of this gene using the morpholino knock-down technology. Loss-of-function phenotypes were analyzed morphologically either at gastrulation or at 24hpf.

Embryos in which FrzA has been inactivated by injection of specific antisense MO develop until early gastrula stage without visible morphological abnormalities. First defects were detected at the end of gastrulation: antisense MO-injected embryos display impairment on the epiboly movement (Fig III-9C). At 11hpf (early somitogenesis, ES), control embryos display a nascent tail bud and the yolk has been completely covered by embryonic cells (Fig. III-9A). In contrast, in antisense MO-injected siblings the yolk plug is not yet closed for embryos injected with high amount of antisense MO (Fig. III-9C). The embryonic axis is shorter for embryos injected with high or medium amount of antisense MO (Fig III-9C ,Fig III-9E) and the somites extend more ventrally (Fig. III-9C). This phenotype is similar to phenotypes of mutants, such a the *knypek* (*kny*) and *trilobite* (*tri*) mutations known to affect convergence-extension movements (Marlow et al., 2002; Sepich et al., 2000; Solnica-Krezel et al., 1996). Similarly to FrzA antisense MO knock-down, these mutants display short body axis and broader somites.

At 24hpf, based on morphological observations, FrzA loss-of-function phenotypes can be sorted out into two classes depending on the MO dose injected and severity of their defects. Embryos injected with the medium dose of antisense MO have a relatively wild-type head shape but display alterations in trunk mesenchyme (Fig. III-9F) and curled tail while the notochord became more obvious and even appeared enlarged. Embryo injected with higher amount of antisense MO displayed a disorganized shape (Fig. III-9D).

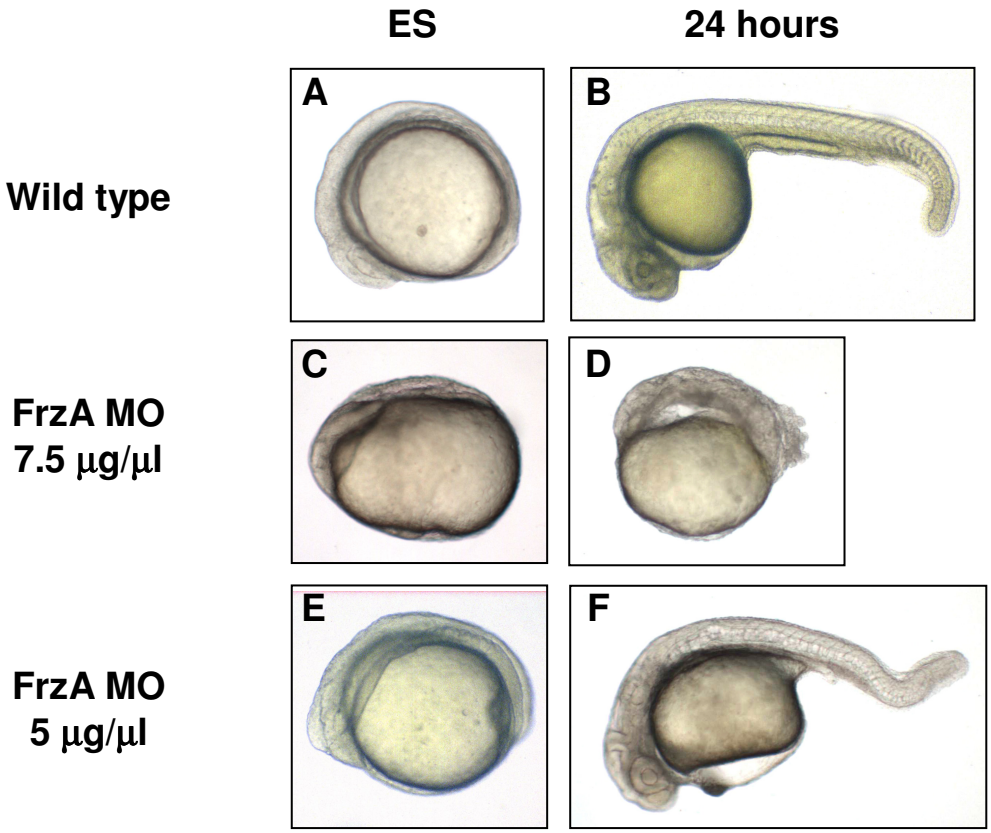


Figure III-9 : FrzA loss-of-function phenotypes
The embryos were injected with FrzA antisense MO at middle (5 µg/µl) or high (7.5 µg/µl) concentration at one cell stage and observe the phenotype at early somitogenesis (ES) or 24 hours post fertilization stage. High concentration FrzA antisense MO impaired epiboly movement can be seen at ES stage (C) and these embryos did not pass through somitogenesis and displayed a ball-like shape at 24 hours stage (D) compare to wild type embryos (A, B). Medium dose of FrzA antisense MO injection caused shorter axis at ES (E) and ventrally curved tail at 24 hours stage (F) but head formation is relatively normal.

Analysis by FrzA overexpression

The activity of FrzA protein was further analyzed during early developmental stages by injecting embryos at the 1-2 cell stage with sense mRNA coding for FrzA (1ng/embryo). No abnormalities were observed during blastula stages and for the epiboly movement during gastrula stage. At 24hpf, the injected embryos (n=192) can be separated into three groups as wide-type like (Fig III-10A, 33%), embryos with a shorter and curled axis (Fig III-10B, 56 %) or embryos truncated in their posterior part (Fig III-10C, 11%). Both groups in B and C display cyclopia phenotypes (Fig. III-10D). These phenotypes are similar to what has been observed after FrzA misexpression in *Xenopus* embryos (Xu et al., 1998). Such a phenotype suggests that FrzA misexpression perturbs normal cellular movements during gastrulation, and therefore, prevents normal axis elongation, leading to a short and bent axis or to a truncated axis.

After FrzA inhibition or overexpression, the trunk and tail formation were both affected and became shorter or absent. However, the causes of these defects could come from different origins, either affecting patterning or cell migration which can both lead to these phenotypes.

As a result, in order to see the reasons that cause the abnormal phenotype, I performed additional experiments to analyse the phenotypes using *in situ* hybridization. I looked at possible effects on the patterning of the embryo using markers related to BMP signalling or to zygotic Wnt8 signalling (which is involved in the control of anterior-posterior axis formation).

(2) Analysis at early developmental stages with tissue specific molecular markers

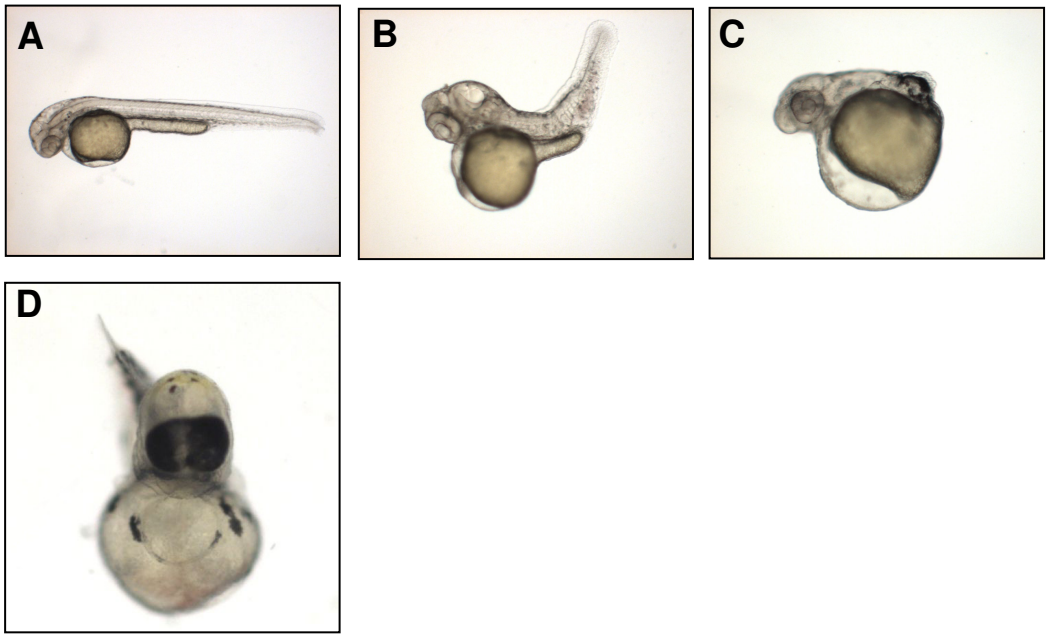


Figure III-10 : FrzA overexpression phenotype at 24 hours. The injection was done with 1 ng/embryo FrzA RNA at 1 cell stage. At 24 hours, the injected embryos can be separated into three groups. (A) wild type like, (B) embryo with curled tail and shorter trunk, (C) embryo without posterior part of the body but has relatively normal head. Both embryos in (B) and (C) have cyclop phenotype which showed the deficiency of eye separation (D). The embryos are either lateral view (A-C) or front view which dorsal to the top (D).

I investigated these hypotheses through the analysis of the expression of various molecular markers at gastrula stage in embryos injected with antisense MO (loss of function) or overexpressing FrzA after injection of sense RNA (gain of function). The different markers used are the bmp antagonist factor chordin, the ventrally expressed transcription factors ved, vent, vox and evel as well as dorsally expressed transcription factors foxA3 and flh (Imai et al., 2001; Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000; Melby et al., 1996; Seiliez et al., 2006).

Analysis of different markers revealed that expression of the BMP antagonist factor Chordin extended toward the ventral part both at the sphere and the shield stage (Fig. III-11 A5-A6, B5-B6) when FrzA was knocked-down. In contrast, Chordin expression was decreased both at sphere and shield stages when FrzA was overexpressed (Fig. III-11 A3-A4, B3-B4). Expression of other components of the bmp pathway, such as the BMP themselves (BMP2b, BMP4) and Ogon (the zebrafish sizzled, known to be antagonist of tolloid (Muraoka et al., 2006) were decreased after FrzA knock down (Fig. III-11 M1-M2, N1-N2, O1-O2, P1-P2) whereas BMP4 expression strongly increased after FrzA overexpression (Fig. III-11Q1-Q2). The expression of a ventral marker Ved, strongly expanded after FrzA overexpression while it almost disappeared after FrzA inhibition (Fig. III-11 C3-C6, D3-D6). On the other hand, the expression of the downstream zygotic Wnt8 target, Vent, did not appear to be changed after either up- or down-regulation of FrzA (Fig. III-11 E3-E6) while the expression of another wnt8 target gene, Vox was down-regulated in FrzA overexpression conditions and a bit up-regulated in FrzA knock down embryos (Fig. III-11 F3-F6).

The data from the analysis of the expression of the zygotic Wnt8 target genes are not consistent with previous reports that suggest FrzA acts by

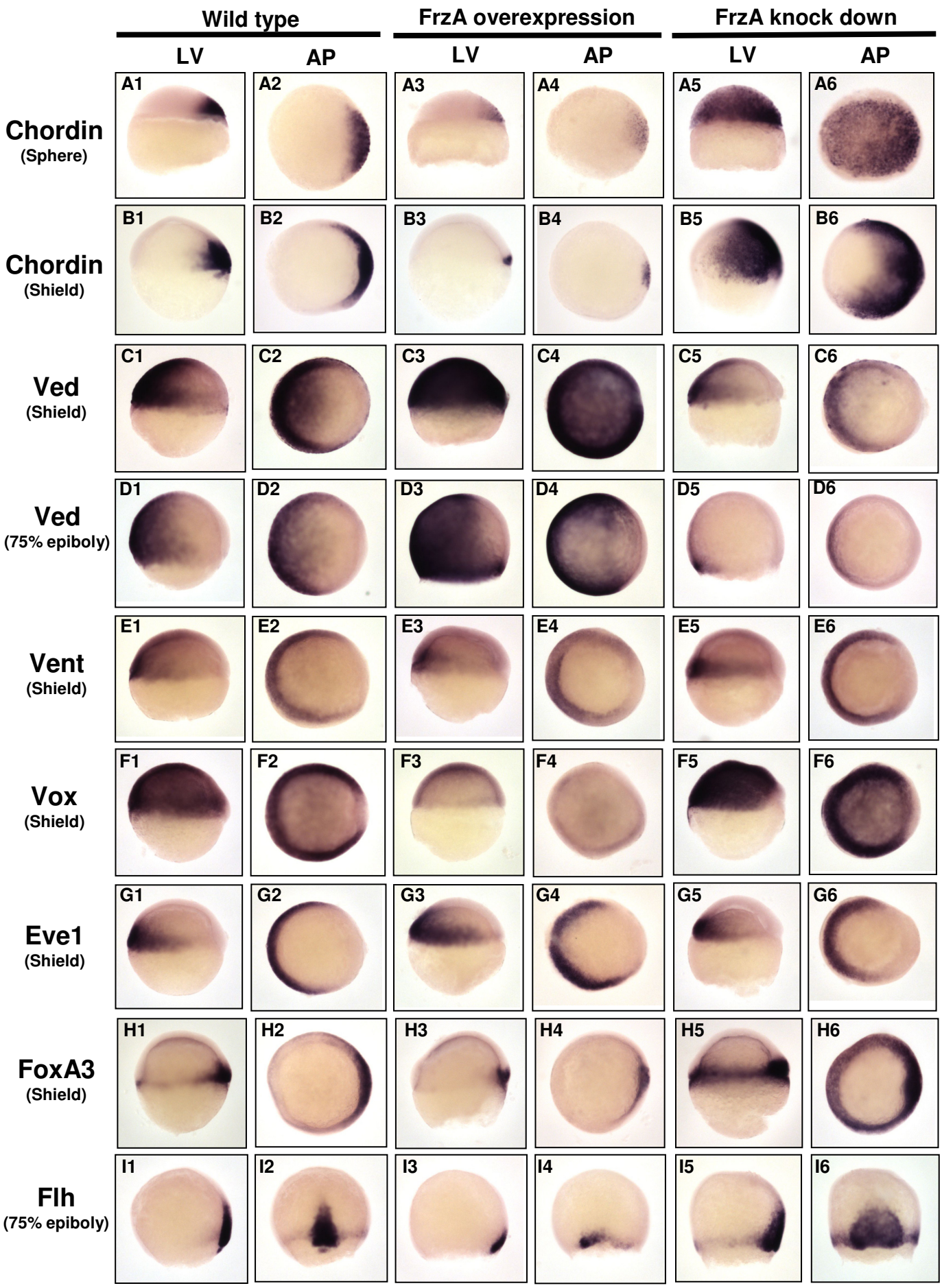


Figure III-11: The expression of different markers upon up- or down- regulation for the FrzA. Different column indicates lateral view (LV) or animal pole view (AP) except I2, I4 and I6 are Dorsal view and N1 and N2 are animal pole view. The columns are separated into three or two treatments, the wild type, the FrzA overexpression and the FrzA knock down. The different markers and the developmental stage for each row are indicated on the left.

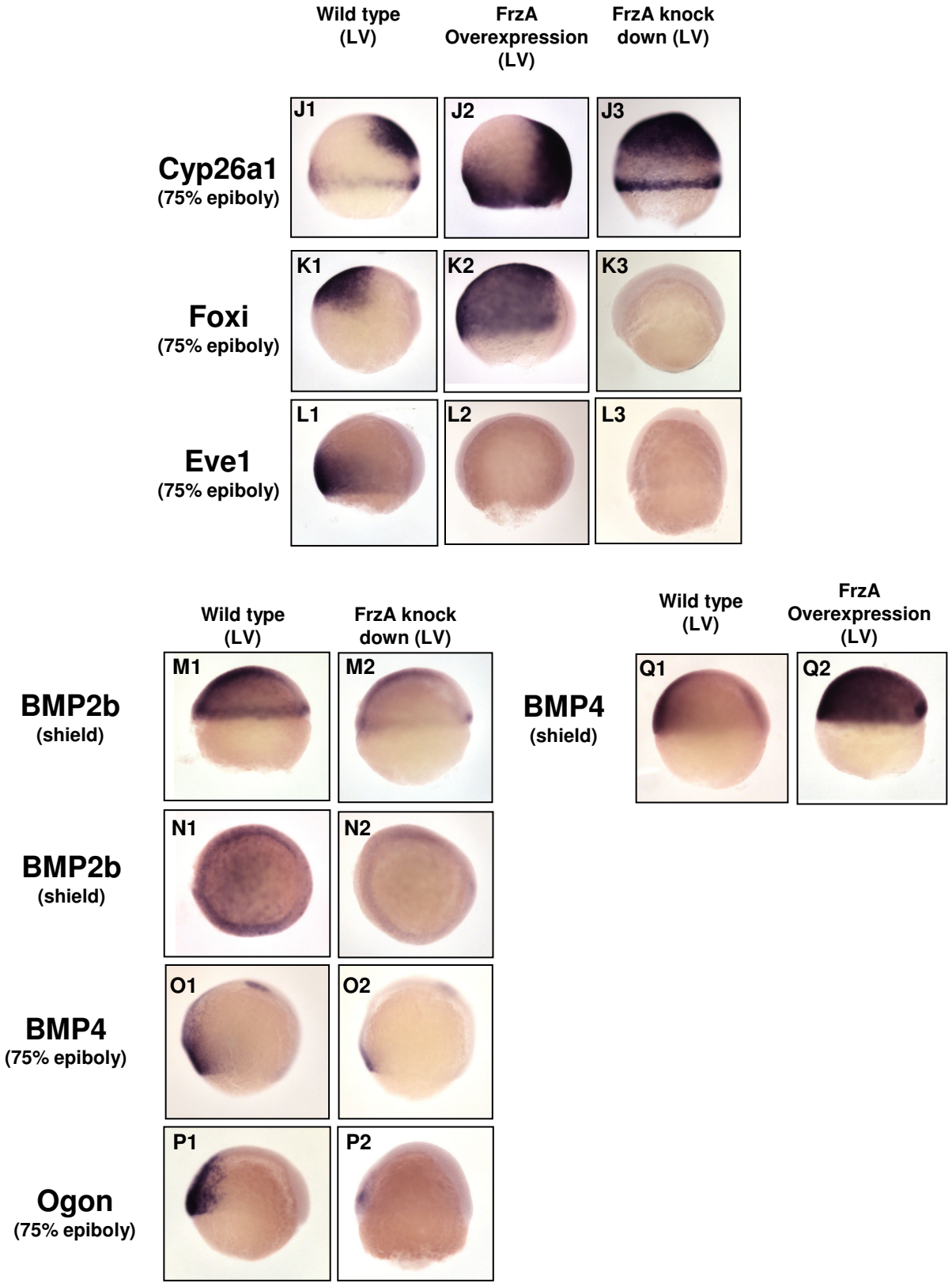


Figure III-11 continue: The expression of different markers upon up- or down- regulation for the FrzA. Different column indicates lateral view (LV) or animal pole view (AP) except I2, I4 and I6 are Dorsal view and N1 and N2 are animal pole view. The columns are separated into three or two treatments, the wild type, the FrzA overexpression and the FrzA knock down. The different markers and the developmental stage for each row are indicated on the left.

binding and inhibiting the zygotic function of Wnt8. Indeed, an inhibition of wnt8 should result in decreasing both Vent and Vox expression while gain of wnt8 function should increase the expression of these two genes. In the present results only Vox expression is in agreement with this hypothesis while this is not the case for Vent. In addition, if FrzA acts a Wnt8 inhibitor, overexpression of this factor should decrease zygotic Wnt activity and in consequence downregulate ventral transcription factors that inhibit ventral chordin expression. Therefore we should observe expansion of chordin in embryos overexpressing FrzA while our data show clearly that chordin expression is strongly downregulated.

According to a previous report, Ved expression appears more under the control of BMP signalling rather than under the control of zygotic Wnt signalling (Gilardelli et al., 2004). In addition, Ved has been shown to be able to repress dorsal genes such as chordin. In our FrzA overexpression we observe an up-regulation of Ved expression and an opposite down-regulation after FrzA knock-down.

Based on these observations it appears that FrzA overexpressing embryos have a higher BMP signalling (more Ved and less chordin) while FrzA knocked-down embryos have lower BMP signalling (less ved and more chordin). In addition, we observed a strong upregulation of bmp4 expression after FrzA overexpression while there is a clear decrease of bmp2b expression in FrzA loss of function experiments. (Fig. III-11 M-O2).

Previous studies have reported that the expression of BMP2b is negatively regulated by Dharma/boz (Leung et al., 2003) a transcription factor which is a downstream target of maternal Wnt signalling. This suggests that the maternal Wnt signalling may be up-regulated in FrzA knocked down embryo.

In addition to the analysis of the different targets of bmp and wnt signalling pathways I also analysed the expression of dorsal specific molecular markers that label the axial mesoderm FoxA3 (Forkhead box A3, (Seiliez et al., 2006)) and Flh (Floating Head, (Melby et al., 1996)). I observed an extension of the expression of these markers toward the ventral side of the embryo (Fig. III-11 H5-H6, I5-I6) after FrzA knock-down and a decrease of FoxA3 and Flh expression after FrzA overexpression (Fig. III-11 H3-H4, I3-I4).

Then I investigated the expression of the ventral marker Eve1. Expression of this gene was not significantly changed after up- or down-regulation of FrzA at the shield stage but eve1 expression disappears at 75% epiboly (Fig. III-11 G3-G6, L1-L3).

For probing the ectoderm, I checked the expression of Cyp26a1 (Kudoh et al., 2002) that labels the anterior neural plate and of Foxi1 (Solomon et al., 2003) that labels the presumptive epidermis. The modification of expression for these two markers in FrzA knock-down embryos is typical of a dorsalization phenotype with loss of ventral territories and ventral expansion of the neural plate. In FrzA gain of function embryos the ventral ectoderm strongly expands dorsally and vegetally while the neural plate territory extended down to the margin (Fig. III-11 K2, J2).

Altogether, knock-down of FrzA leads to dorsalization of the embryos as well as an increase of axial, paraxial mesodermal and dorsal ectodermal territories. This may be due to increased Chordin activity resulting possibly from an up-regulation of the maternal β-catenin activity. In contrast, gene expression analyses in FrzA overexpression experiments show an increase of BMP signalling - that may be resulting from a decrease of chordin activity. To investigate this possibility, I tested whether a decrease of chordin activity could rescue FrzA knocked down embryo.

3. FrzA and the BMP signalling:

a. The phenotype of FrzA inhibition of function can be rescued by decreasing Chordin activity

I tested the previous hypothesis by performing double knock-down of FrzA and Chordin.

As shown in figure III-12, the dorsalization phenotype resulting from FrzA knock-down is rescued when Chordin is inhibited at the same time. The expression of the markers cyp26a1 and Foxi1 were restored to wild type like expression pattern (Compare Fig. III-12B, F and C, G). This rescue was also analysed at the somitogenesis stage using the mesodermal marker MyoD. Dorsalized embryos display a radial expansion of somitic furrows labelled by MyoD. As shown in Figure yy embryos showed circularized MyoD expression after FrzA knock down (Class1, Fig. III-12I) were almost completely rescued (Class 2, Fig. III-12I) by removing Chordin activity.

b. Phenotype of FrzA knock down can be rescued by BMP overexpression

My previous results suggest that the phenotypes resulting from FrzA loss of function is due to excess of chordin. This also suggests that this phenotype is due to lack of BMP activity. To demonstrate that this is the case I injected either FrzA antisense MO alone or FrzA antisense MO plus BMP RNA into one cell stage embryo then I analyzed with the molecular markers Cyp26a1, Foxi1 and Ved at the 75% epiboly stage (mid-gastrulation). The analysis reveals that the ventral ectodermal extension of Cyp26a1 expression in FrzA knocked-down embryos was reduced (Fig. III-13B, C) after BMP co-injection.

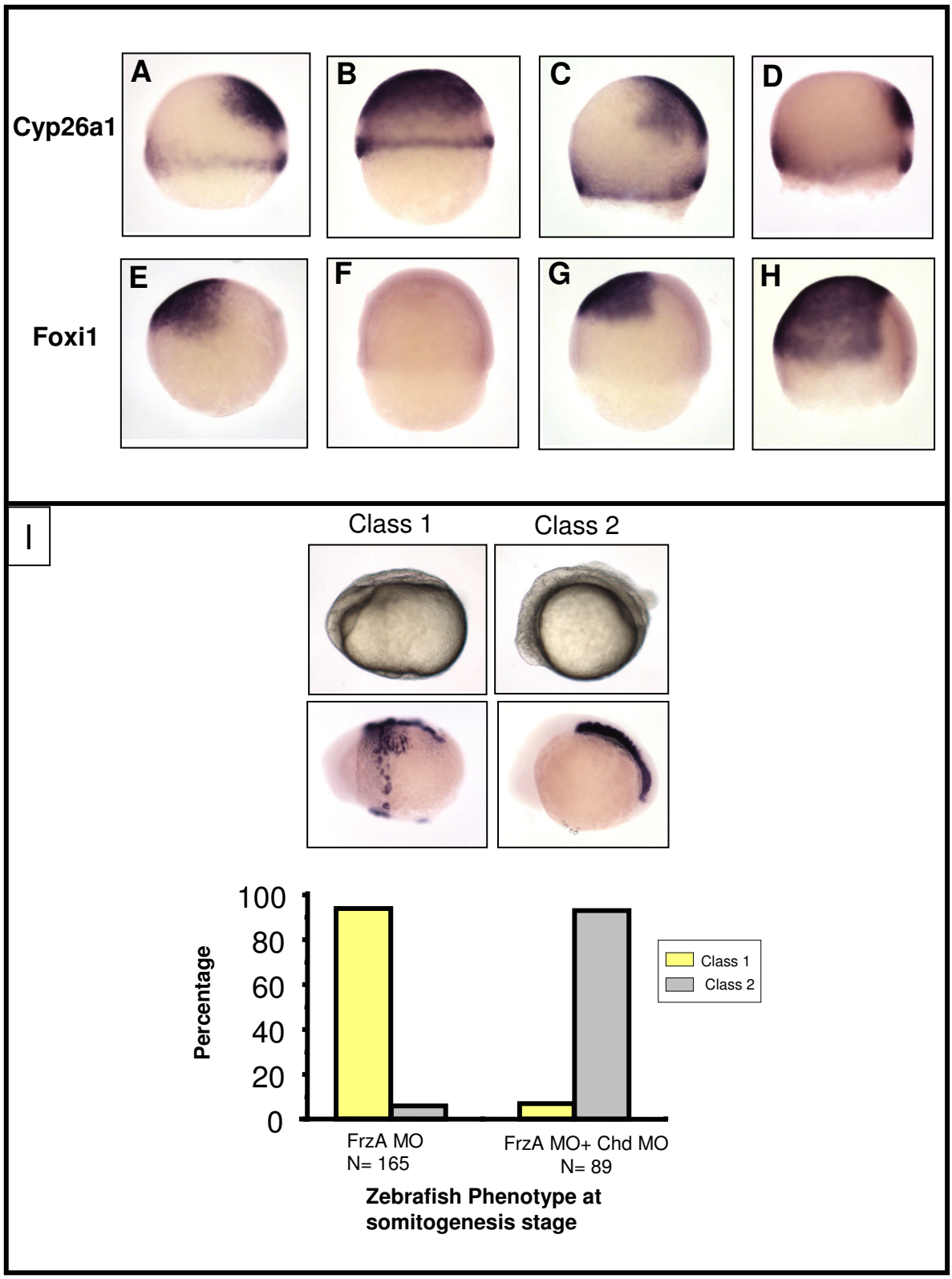


Figure III-12: The rescue of the FrzA knock down defect by the Chordin knock down. The embryos were either wild type (A,E) or injected with FrzA antisense MO alone (B,F) or FrzA antisense MO plus Chordin antisense MO (C,G) or Chordin antisense MO alone (D,H) on the whole embryo until 75% epiboly stage then checked with the presumptive brain marker (Cyp26a, A-D) or presumptive epidermal marker (Foxi, E-H). In all embryos the dorsal part is on the right (A-H). The rescue result can also be detected by the phenotype statistics at somitogenesis stage (I). The FrzA knock down phenotype (Class 1) can be rescued by the Chordin knock down in the same embryo and become more wild type-like phenotype (Class 2).This rescued phenotype not only can be seen in the live embryo (upper layer) but also in the myo D (muscle) *in situ* hybridization staining (lower layer). FrzA MO: the embryo injected with FrzA antisense MO. FrzA MO+Chd MO: the embryo injected with FrzA antisense MO plus Chordin antisense MO.

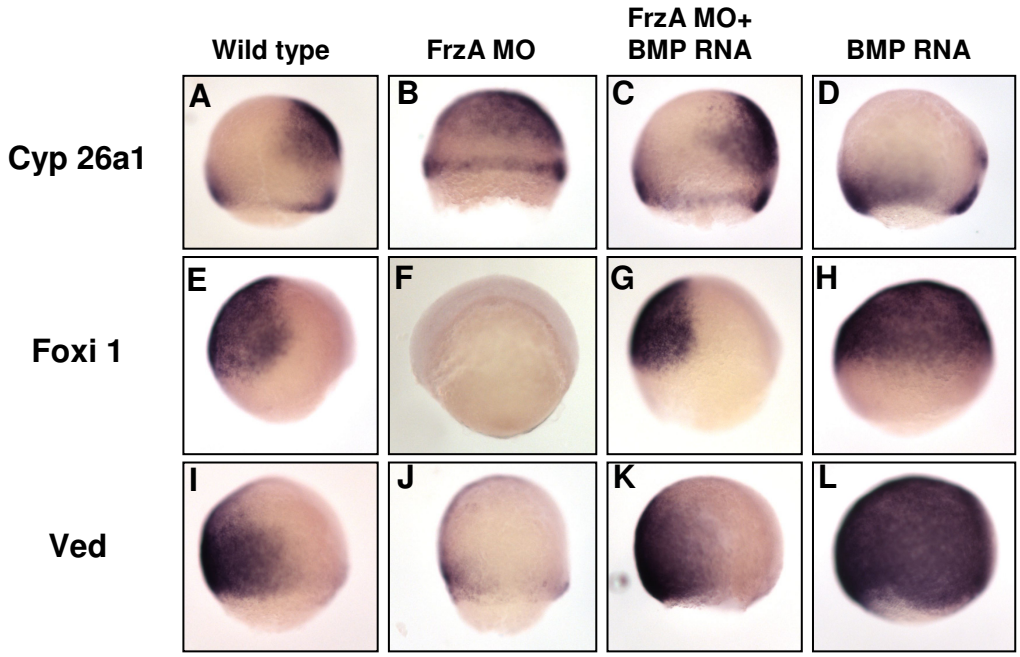


Figure III-13 : The rescue of FrzA knocked down defect by BMP RNA overexpression. The embryos were either wild type (A, E, I) or injected with FrzA antisense MO (B, F, J) or FrzA antisense MO plus BMP RNA (C, G, K) or BMP RNA (D, H, L) on the whole embryo until 75% epiboly stage then checked with the different markers which indicated on the left for each row include Cyp26a1,Foxi 1 and Ved. All the embryos are dorsal to the right and animal pole to the top.

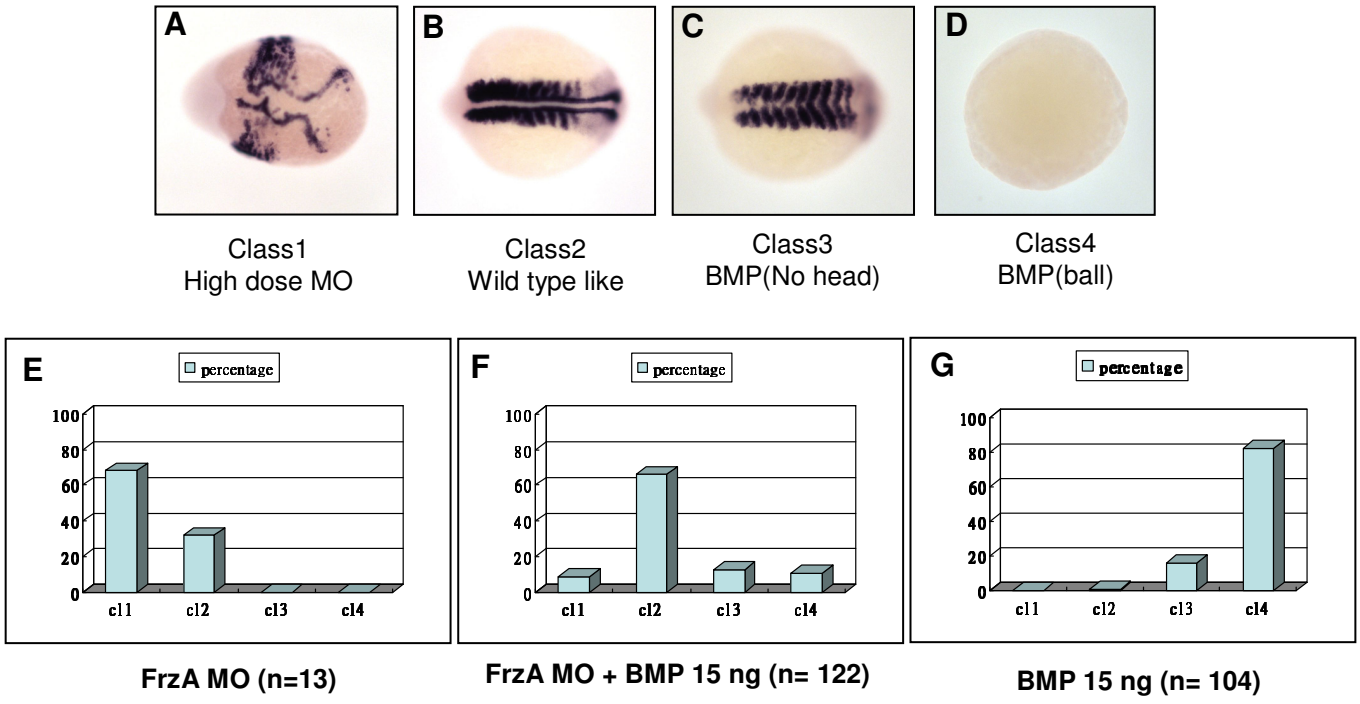


Figure III-14 : The rescue of the FrzA knock down defect by the BMP RNA overexpression and checked by the myoD expression at the somitogenesis stage. The embryos were either injected with FrzA antisense MO (E) alone or FrzA antisense MO plus BMP RNA (F) or BMP RNA alone (G) on the whole embryo at one cell stage until somitogenesis stage then checked with the myoD marker and separated the embryo into 4 classes (Figure A-D) then calculate the percentage for each class. All the embryos are anterior side to the left and dorsal front view. N indicates embryo numbers.

Ventral ectodermal markers Foxi1 and Ved in the FrzA knocked down embryos appeared also rescued when BMP RNA was co-injected (Fig. III-13F, G, J, K). This has to be compared with conditions where BMP RNA is injected alone. In this case, the embryos become ventralized and the expression of Cyp26a1 appear limited to a very small dorsal region or is completely lost while expression domain of the ventral ectodermal marker Foxi1 is strongly expanded. Similarly, expression territory of Ved also strongly increases after injection of BMP RNA.

In addition to the dorsal ventral markers, I also checked the expression of the somitic marker MyoD at somitogenesis stage under the same conditions. The percentage of embryos displaying a circularized MyoD expression after FrzA antisense MO injection decreased from 68% to 9% when BMP RNA was co-injected (Fig. III-14E, F, class 1). Conversely, the percentage of wild type like embryos increased from 32% to 66% (Fig. III-14E, F, class 2). Finally, when BMP RNA is injected alone, most of the embryos loose their MyoD staining (Fig. III-14D, G). Altogether, these observations demonstrate that the dorsalisation phenotypes resulting from FrzA inhibition can be rescued by a concomitant overexpression of BMP at both gastrula and somitogenesis stages. This result further confirms that the BMP signalling pathway is down-regulated after FrzA -inhibition.

In addition to patterning defects, the phenotype of somites enlargement revealed after in situ hybridization with MyoD or the delay in epiboly movement observed for FrzA knocked down embryos at the somitogenesis stage suggests that cell movements are also affected.

c. Effects of FrzA on convergence extension movements

In addition to the patterning defects observed after up- or down-regulation of FrzA activity, I observed alteration of cell movements such as convergence and epiboly movements. This was analyzed by in situ hybridization on whole mount embryos for the expression of tissues specific markers that allow an easy qualitative observation of alteration of cell movements. I checked three movements: the anterior migration of the prechordal plate, the convergence of lateral cells toward the dorsal side of the embryo and the extension of the embryonic axis.

To probe the anterior migration of the prechordal plate under the epiblast I visualized this tissue using Hgg1 (ctsl1b, cathepsin Like 1b). In the wild type situation, the prechordal plate migrates anteriorly (Fig. III-15B, red arrow) and at the end of gastrulation is observed immediately anterior to the neural plate border (Fig. III-15B, green arrow). I analyzed the convergence movement by following the movement of neural plate border cells toward the dorsal side of the embryo. These border cells were visualised using Dlx3 (Distal-less homeoobox gene 3b) and I measured the width of the neural plate (Fig. III-15B, green arrow). Finally the extension of the embryonic axis was monitored by measuring the length of the notochord (Fig. III-15A, C, blue arrow) labelled with ntl (no tail) (Heisenberg et al., 2000).

Performing these experiments I observed that neither knocked down nor overexpression of FrzA embryos can undergo normal cell movements.

The prechordal plate fails to move normally through the neural plate and at the tail bud stage is located posteriorly to the neural plate border both in the FrzA knock down or in FrzA overexpressing embryos (Fig. III-15E, H). In addition, an absence of convergence movement is observed as revealed by Dlx3 labelled neural plate border cells (Fig. III-15D, green arrow) both in FrzA loss of function conditions as well as in FrzA overexpression embryos

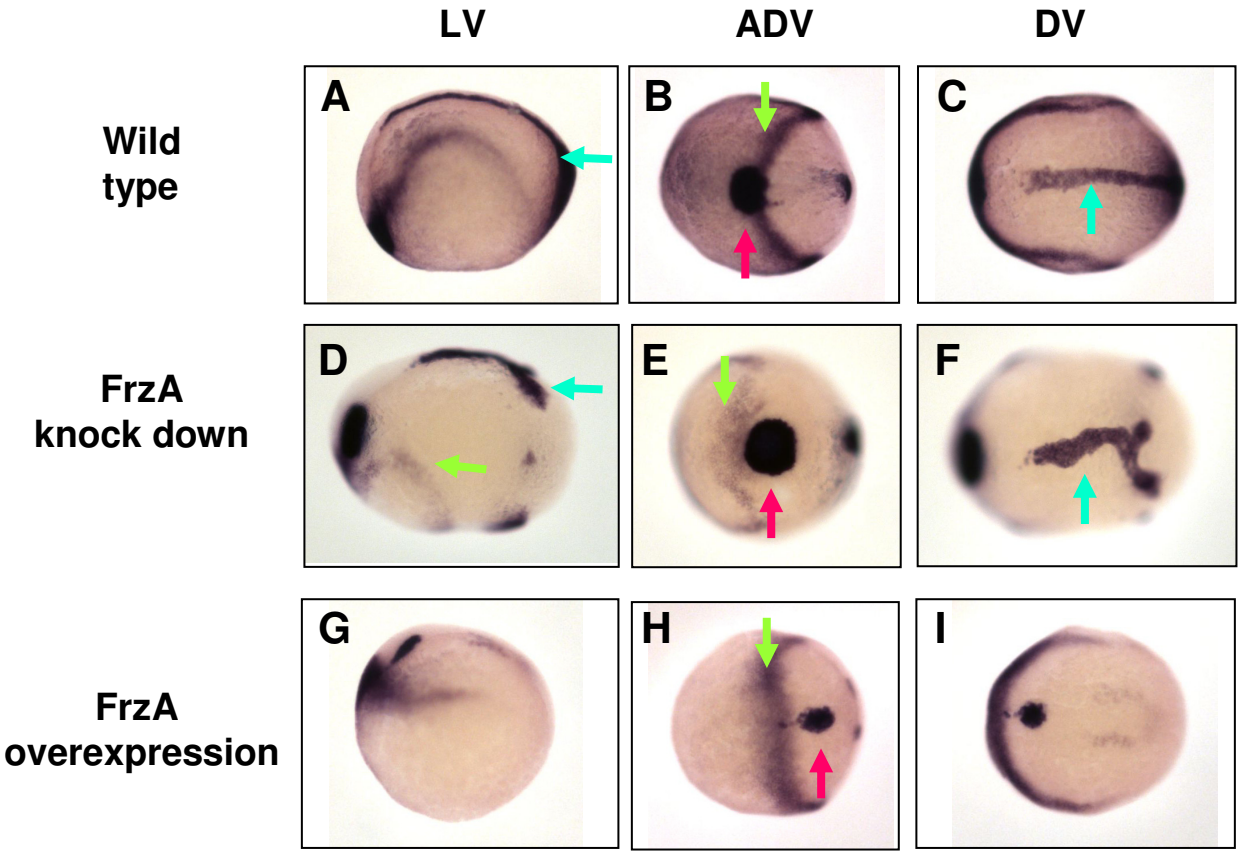


Figure III-15 :The effect of up- or down-regulation of the FrzA on the cell movement. (A-I) The cell movement in the wild type (A-C), FrzA knock down (D-F) or FrzA overexpression (G-I) embryos at tail bud stage were determined by the *in situ* hybridization using three markers, Hgg1(red arrow), No tail (blue arrow) and Dlx3 (green arrow). Different columns indicate different point of view, LV: Lateral view, ADV: Anterior dorsal view, DV: Dorsal view. (A, D, G, C, F,I) Anterior part to the left, (A, D, G) dorsal part is on the top or (C, F, I) dorsal part front view. (B, E, H) anterior part front view and dorsal part to the right.

(compare Fig. III-15B and H, green arrow). For the dorsal extension movements which is measured based on the length of the notochord territory I observed a shorter notochord in FrzA knocked-down embryos indicative of defective extension of the axis (compare Fig. III-15A and D, blue arrow). The absence of notochord in FrzA overexpressing embryos makes it impossible to measure the dorsal extension in FrzA overexpression conditions.

In conclusion, up- or down-regulation of FrzA does not only affect the embryo patterning but also affect cell movements. I tried to quantify the extent of the effect on the convergence and extension movements by following over time in a live embryo the convergence of labelled cells as well as the length of the embryonic axis.

To perform these experiments I used a particular green fluorescent protein (Kaede) which can be converted from green to red upon irradiation at a particular wavelength (see procedure section in part III of the thesis). Kaede RNA was co-injected with either a control MO or an FrzA antisense MO into the embryo at the one cell stage and, at the onset of gastrulation (shield stage) a small group of cells were irradiated to photoconvert the green fluorescence of the Kaede protein into red fluorescence. For the convergence movement, the cells irradiated are in lateral position at an angle of 90° from the dorsal midline and the movement is measured over time by the decrease of the angle between labelled cells and the dorsal midline. For the measurement of the extension movement, the group of cells examined correspond to the dorsal margin (the shield) and the measure is made on the length of the axis over time.

When comparing wild type conditions (Kaede injected alone) with injection of a control MO (Fig. III-16A-C, green line in Fig G) or with a FrzA antisense MO (Fig. III-16D-F, red line in Fig. III-16G) I observed that the dorsal extension

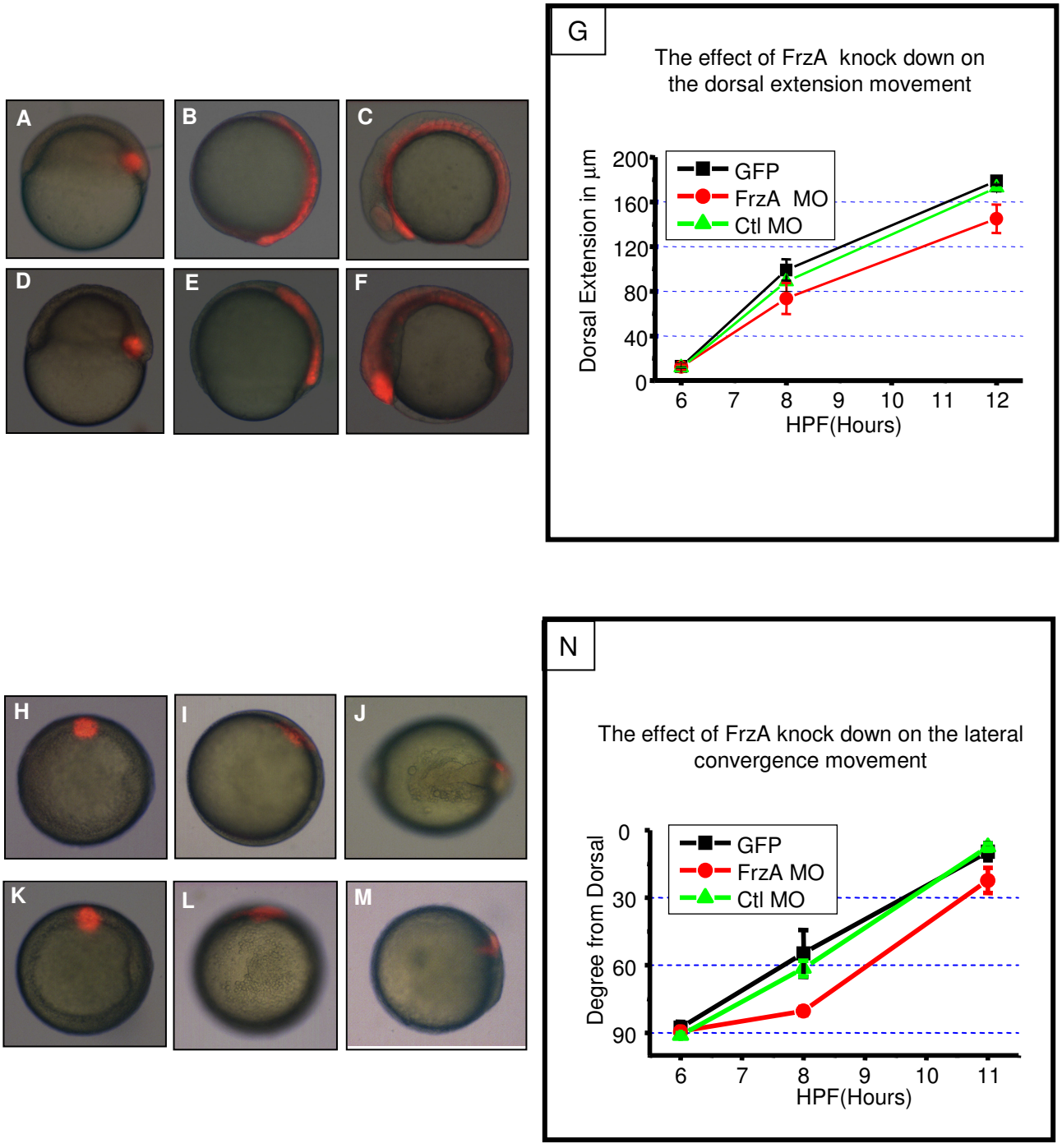


Figure III-16 :The effect of FrzA knock down on the dorsal extension and the convergence movement. The embryos were injected with the GFP(A-C, H-J) as a control or the FrzA antisense MO (D-F, K-M) then measured the length of the labeled axis or the angles between the labeled cell and the dorsal side at different developmental stages, respectively. The movements of dorsal extensions (G) and convergences (N) of different treatments include GFP RNA, control MO (Ctl MO) and FrzA antisense MO are calculated. Comparing to control treatments (black and green line), FrzA inhibition embryos (red line) can not undergo both normal dorsal extension and convergence movements. (A, D,H, K) shield stage, (B, E, I, L) Tailbud stage, (C, F, J, M) Somitogenesis stage. (A-F) Lateral view and (H-M) Animal pole view. The dorsal site is on the right in the all embryo except (C, F) which the dorsal side is on the top.

was already significantly retarded at 8 hpf (hours post fertilization), 2 hours after photoconversion, and this was still true at 12 hpf. For the convergence movement I observed a significative effect, in FrzA loss of function conditions (Fig. III-16K-M, Fig. III-16N red line compared to the control MO injection, Fig. III-12H-J, Fig N green line) at 8 and 11 hpf. As I already mentioned, both knock down or overexpression of FrzA may affect the BMP signalling and in consequence influence cell movements. Nevertheless, the mechanism by which FrzA regulates the BMP signalling is not clear yet but may be due to the effect on Chordin expression.

The up-regulation of chordin expression in the FrzA knocked down embryos may due to 2 possible mechanisms. It may be directly induced by the maternal Wnt signalling, or it may be due to a decrease of Vent andVox expressions which are both known to repress chordin transcription and which are under the control of the zygotic Wnt8 signalling. However, this second hypothesis is rather unlikely because Vent and Vox expression are not reduced after FrzA knockdown. Nevertheless I would need more evidence to be able to rule out the possibility of an implication of the zygotic Wnt8 signalling.

4. Comparison of function of FrzA with the function of known Wnt 8 inhibitors, FrzB and DKK1:

In order to clarify the relationship between Wnt8 and FrzA, I compared the effect of FrzA knock-down with the effect of loss of function of the well known Wnt8 signalling inhibitors FrzB and Dickkopf1 (DKK1).

a. Comparative analysis of FrzA, FrzB and DKK1 loss of function phenotypes

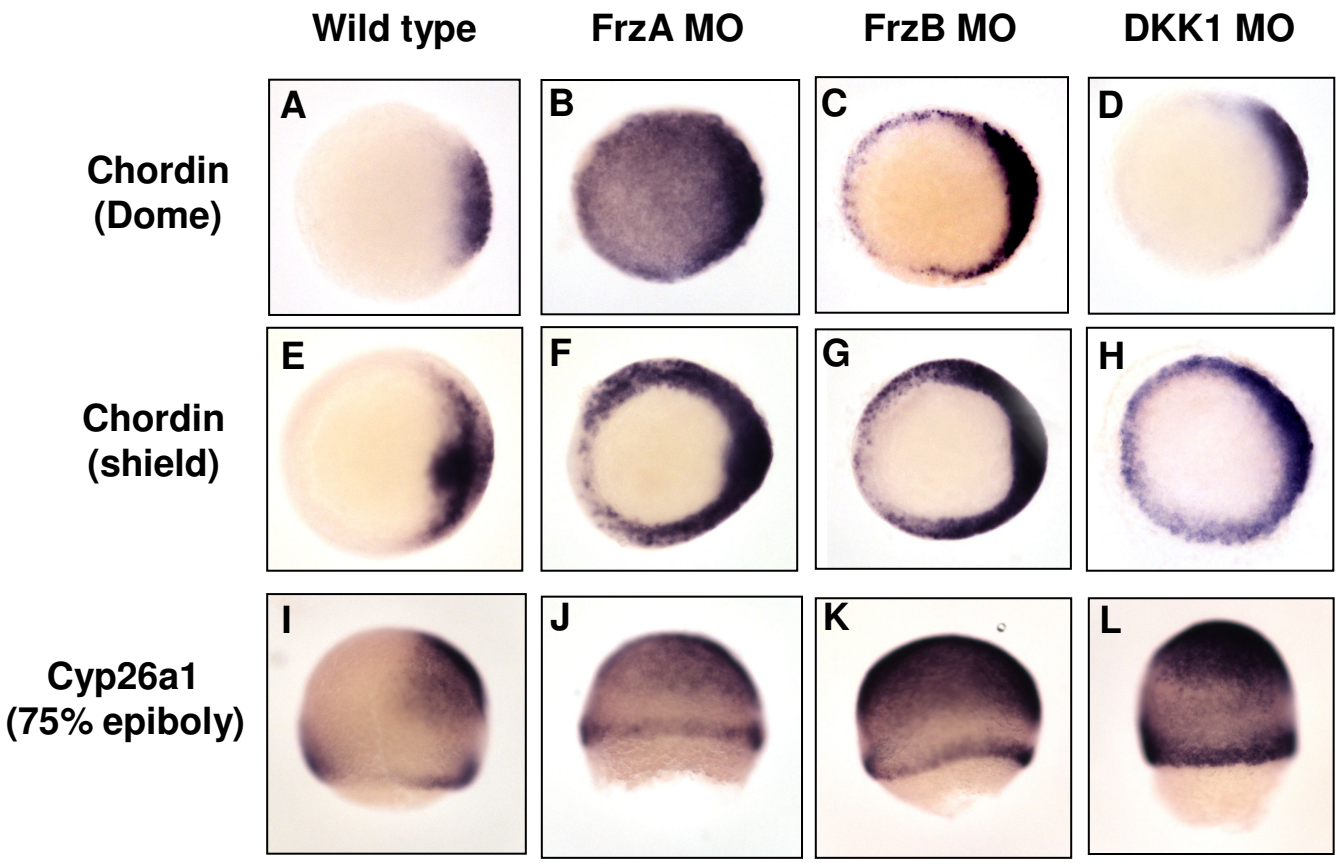


Figure III-17: Comparing the chordin and cyp26a1expression in the different Wnt signaling inhibitors (FrzA, FrzB and DKK1) knocked down embryo. (A-H) are animal pole view and (I-L) are Lateral view, all the embryos dorsal side to the right. The labels above each column indicate the injected antisense MO and the labels on the left side of each row indicated different *in situ* hybridization markers and the developmental stages. All the injected embryos shows increase of chordin expression and typical dorsalization which the expression of Cyp26a1 cover the whole ectoderm.

At first I compared phenotypes of FrzA, FrzB and DKK1 loss of function embryos. I injected antisense MOs corresponding to these three genes and checked the expression of chordin and of Cyp26a1.

I observed that the expression of chordin increased both in FrzB and DKK1 antisense MO injected embryos starting from the dome stage (FrzB antisense MO) or from the shield (DKK1 antisense MO) stage (Fig. III-17C, G, H). The delayed increase of chordin expression in DKK1 knock-down embryo is likely due to its absence of expression at early blastula stage (Fig. III-17D and Fig. III-5).

For Cyp26a1, both FrzB and DKK1 knock-down embryos showed typical dorsalization phenotype (Fig. III-17K, L) although domains of expression are a bit different (FrzB knocked down embryos showed wider Cyp26a1 expression than the DKK1 knocked down embryos).

From the chordin and Cyp26a1 staining I hypothesized that FrzB and DKK1 knock-down embryos may reveal a decrease of BMP signalling similar to FrzA knock-down embryos.

b. Rescuing effect of chordin loss of function and of BMP overexpression on FrzA, FrzB and DKK1 knock-down embryos

To test the previous hypothesis, I tried to rescue FrzB or DKK1 antisense MOs injected embryos in conditions that allow increasing the BMP signalling, coinjecting either Chordin antisense MO or BMP RNA.

Injections were performed at one cell stage and the embryos were analyzed at the 75% epiboly with different molecular markers: Cyp26a1, Foxi1 and Ved. Results showed that the dorsalization phenotype of FrzB knock down embryos can be rescued by either Chordin antisense MO (Fig. III-18A-L) or

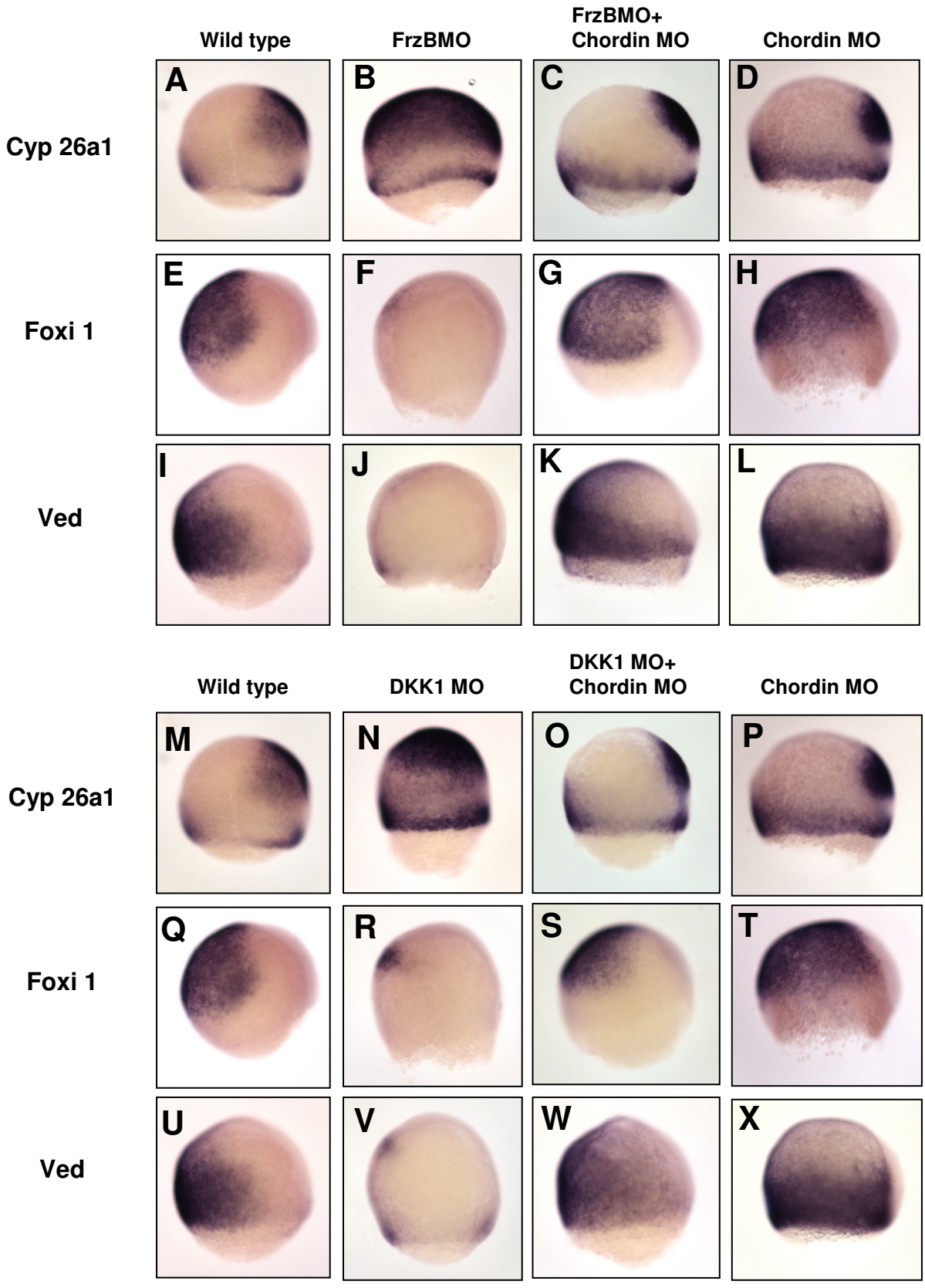


Figure III-18: The rescue of the FrzB and Dickkopf 1 (DKK1) knocked down defect by the Chordin knock down. The embryos were either wild type (A, E, I, M, Q, U) or injected with FrzB antisense MO (B, F, J) or DKK1 antisense MO (N, R, V) or FrzB antisense MO plus Chordin antisense MO (C, G, K) or DKK1 antisense MO plus Chordin antisense MO (O, S, W) or Chordin antisense MO (D, H, L, P, T, X) on the whole embryo until 75% epiboly stage then checked with the different markers which indicated on the left for each row include Cyp26a1,Foxi 1 and Ved. All the embryos are dorsal to the right and animal pole to the top.

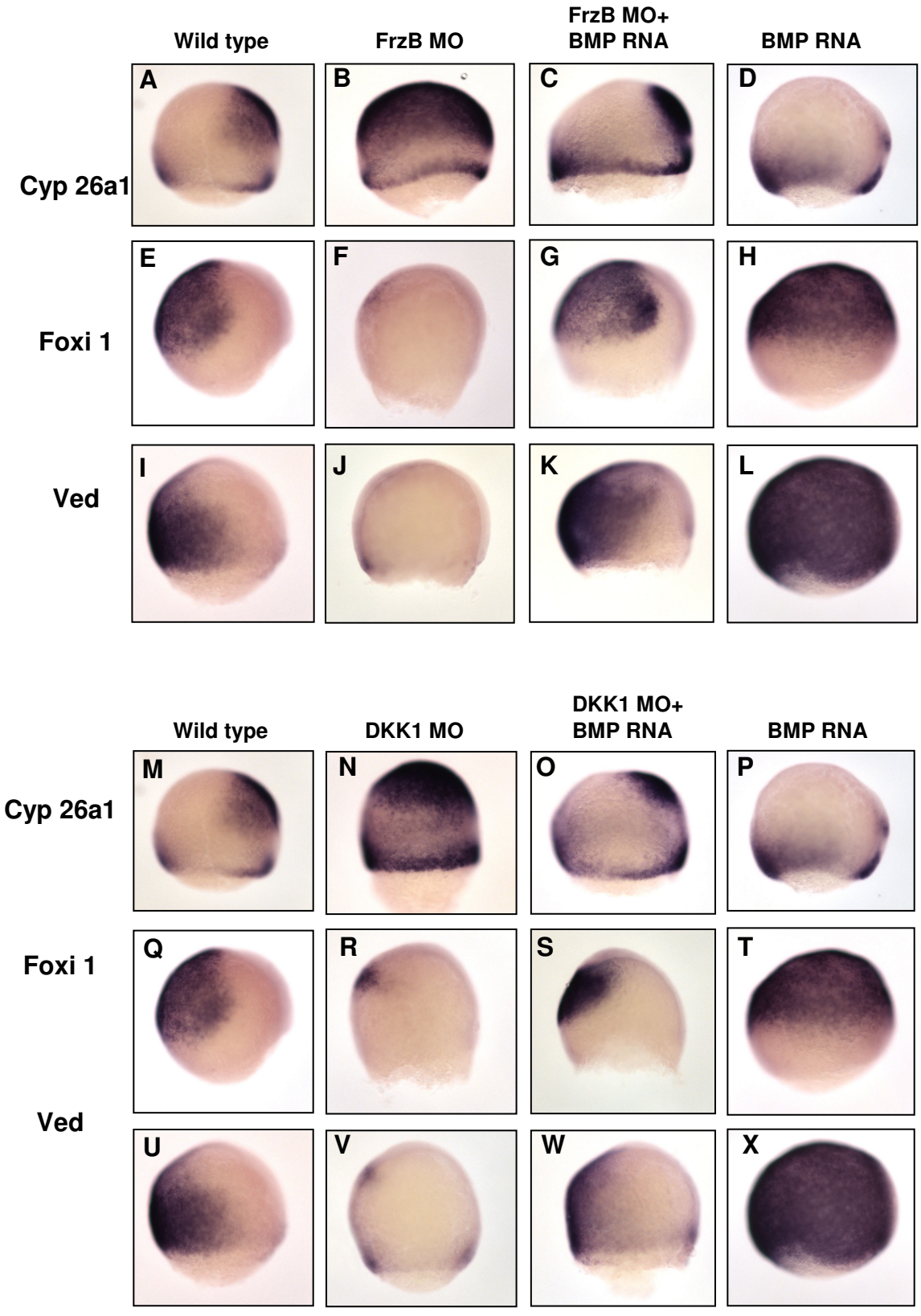


Figure III-19: The rescue of the FrzB and Dickkopf 1 (DKK1) knocked down defect by the BMP overexpression. The embryos were either wild type (A, E, I, M, Q, U) or injected with FrzB antisense MO (B, F, J) or DKK1 antisense MO (N, R, V) or FrzB antisense MO plus BMP RNA (C, G, K) or DKK1 antisense MO plus BMP RNA (O, S, W) or BMP RNA alone (D, H, L, P, T, X) on the whole embryo until 75% epiboly stage then checked with the different markers which indicated on the left for each row include Cyp26a1,Foxi 1 and Ved. All the embryos are dorsal to the right and animal pole to the top.

either overexpressing BMP (Fig. III-19A-L). In rescued embryos the ectodermal Cyp26a1 expression appeared restricted to the dorsal side (Fig. III-18B, C and Fig. III-19B, C), the Foxi1 expression in the ventral ectoderm was detected again (Fig. III-18F, G and Fig. III-19F, G) the expression of the ventral marker Ved which disappeared in the FrzB morphant was detected again when the BMP signalling was up-regulated either after Chordin inhibition (Fig. III-18J, K) or after BMP RNA overexpression (Fig. III-19J, K).

A rescue of the dorsalization phenotype can also be seen for DKK1 knocked down embryos after either Chordin knock-down (Fig. III-18M-X) or BMP overexpression (Fig. III-19M-X). In the rescued embryos, the ventral ectodermal expression of Cyp26a1 was restricted to the dorsal side and replaced by Foxi1 expression when the BMP signalling was up-regulated after Chordin knocked down (Fig. III-18N, O, R, S) or after BMP RNA overexpression (Fig. III-19N, O, R, S). The ventral Ved expression which appears decreased in the dorsalized embryos increased again when BMP signalling was upregulated (Fig. III-18V, W and Fig. III-19V, W).

In summary, rescue experiments showed that up-regulation of BMP signalling can rescue the dorsalisation phenotype observed in FrzB and DKK1 loss of function conditions. This suggests that the effect of FrzB, DKK1 and FrzA on the dorsoventral patterning is mediated through the regulation of Chordin activity likely by the same mechanism. Based on this data, these three factors appear to function in a similar way.

c. Comparative analysis of FrzA, FrzB and DKK1 overexpression phenotypes

In order to test the last hypothesis I compared the gain of function phenotypes for these three genes.

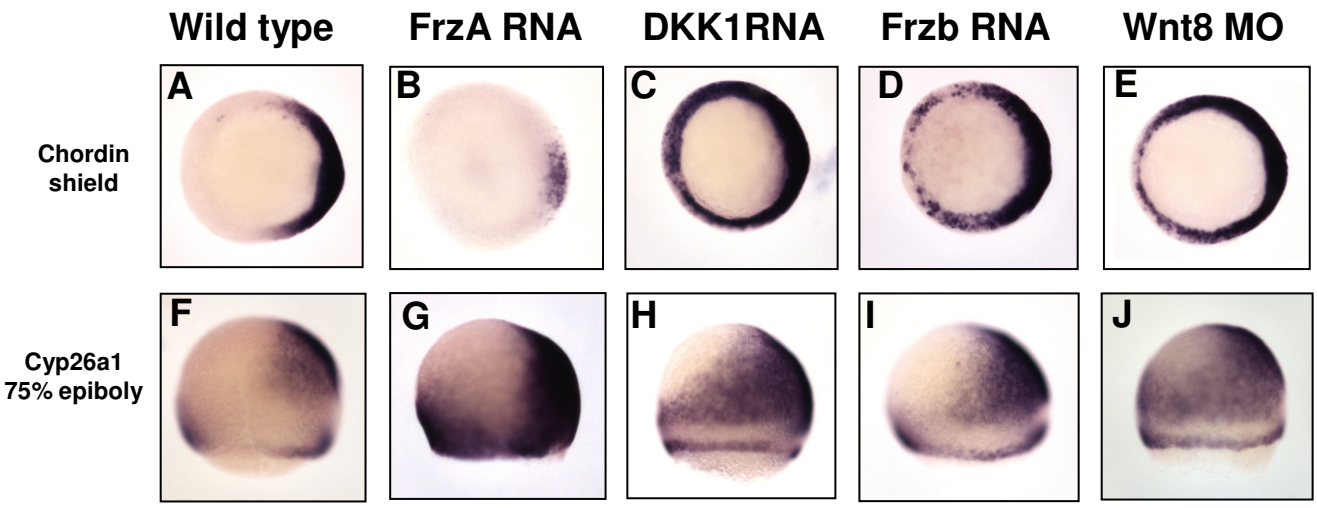


Figure III-20: The chordin and Cyp26a1expression in the FrzA, FrzB and DKK1 overexpression and Wnt8 knocked down embryos. (A, F) Wild type embryo, (B, G) FrzA RNA injected embryo, (C, H) DKK1 RNA injected embryo, (D, I) FrzB RNA injected embryo, (E, J), Wnt8 morpholino antisense RNA injected embryo. (A-E) chordin staining at shield stage and dorsal view. (F-J) cyp26a1 staining at 75 % epiboly stage and lateral view. All embryos are dorsal to the right. Overexpression of known Wnt8 antagonists include DKK1 and Frzb show similar patterning effect as Wnt8 inhibition but not FrzA overexpression.

In order to know whether FrzA acts as a Wnt8 signalling inhibitor, I compared the phenotypes resulting from the overexpression of FrzA, FrzB and DKK1 as well as with the phenotype of Wnt8 antisense MO knock down. Analysis of embryos from these experiments has been performed at 75% epiboly using the chordin and cyp26a1 markers.

Since the FrzB and DKK1 have previously been shown to act as Wnt8 signalling inhibitors (Brott and Sokol, 2002; Wang et al., 1997a), their overexpression phenotype should be similar to the Wnt8 knock down. In good agreement with this hypothesis, the expression territories of chordin are the same in the FrzB, DKK1 overexpression and Wnt8 morphants embryos and appear expressed in the whole gastrula margin (Fig. III-20C-E).

Surprisingly, overexpression of FrzA generates a different phenotype consisting of a strong reduction of Chordin expression (Fig III-20B). I observed also difference in the expression of Cyp26a1 between embryos overexpressing FrzA and those overexpressing FrzB or DKK1. The expression territories are the same in FrzB and DKK1 overexpressed embryos as well as in Wnt8 morphants embryos and are characteristic of an anteriorization of the embryo (Fig. III-20H, I, J). However, in FrzA overexpressing embryos the expression of Cyp26a1 increases in intensity and extend dorsally down to the margin. (Fig. III-20G).

Therefore comparison of gain of function experiments for FrzA, FrzB and DKK1 suggests that the molecular mechanism of action of FrzA is different from those of FrzB and DKK1. In particular it appears that FrzA is unable to inhibit the zygotic wnt8 signalling.

d. Rescue of FrzA loss of function by FrzA, FrzB and DKK1 RNA injection

To see if FrzB or DKK1 functions are different from FrzA function, I performed a series of rescue experiments where I knocked down the FrzA expression using a 5'UTR FrzA antisense MO that inhibits the translation of the transcript but would not block the function of an FrzA RNA that does not carry the same 5' UTR. Then I tried to rescue these morphants by injection of either a 5' UTR FrzA antisense MO with a synthetic FrzA RNA or either with FrzB or DKK1 RNA. The rescue phenotypes were analyzed by looking at the expression of chordin at the onset of gastrulation. As expected the FrzA RNA can rescue the FrzA knock-down phenotype (Fig. III-21E). At the opposite, neither FrzB (Fig. III-21D) nor DKK1 (Fig. III-21C) RNA are able to rescue FrzA morphants. This strongly argues in favour of the hypothesis that FrzA acts through a different molecular mechanism than FrzB and DKK1.

All data collected so far are in favour that FrzA may be involved in the regulation of maternal Wnt signalling regulation but not of zygotic Wnt8 signalling. In addition, FrzA overexpressing embryos show up-regulated BMP signalling and the molecular mechanism of action is likely to be different from the mechanism known for the other Wnt inhibitors (FrzB and DKK1).

e. The connection of FrzA, FrzB and DKK1 to Wnt signalling

From the previous experiments, questions on the function of FrzA are still open. Would this factor really regulate the Wnt signalling and which type (maternal vs zygotic) has still to be demonstrated. To approach this question, I looked at the expression of a read out of the Wnt signalling pathway, Axin2, in FrzA, FrzB and DKK1 knock-down embryos. Axin2 is involved in the axin complex which decreases the Wnt signalling through phosphorylating the β-catenin (Bienz, 2005) and has been reported to act as a feedback inhibitor of Wnt signalling (Leung et al., 2002; Lustig et al., 2002).

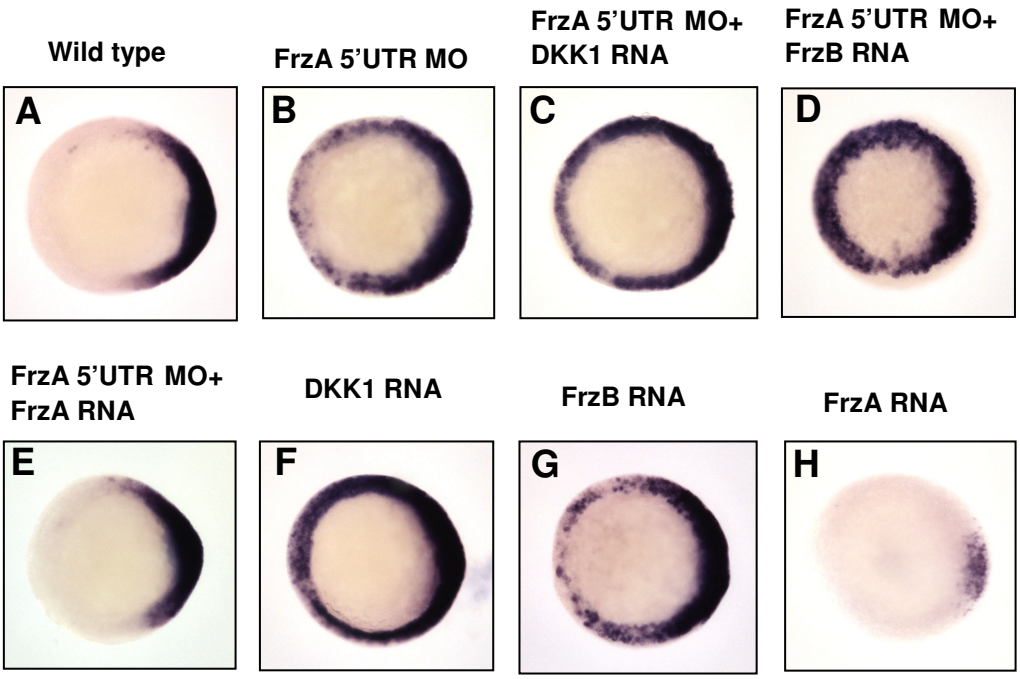


Figure III-21: The FrzA acts not the same way as the other Wnt inhibitors and is not the redundant gene. The embryos were injected with FrzA 5'-UTR antisense MO (B) or with FrzA 5'-UTR antisense MO plus Dickkopf 1 RNA (C) or with FrzA 5'-UTR antisense MO plus FrzB RNA (D) or FrzA 5'-UTR antisense MO plus FrzA RNA (E) or with Dickkopf 1 RNA alone (F), FrzB RNA alone (G), FrzA RNA alone (H). All the embryo are at the shield stage and staining with Chordin and from the animal pole view, dorsal part to the right.

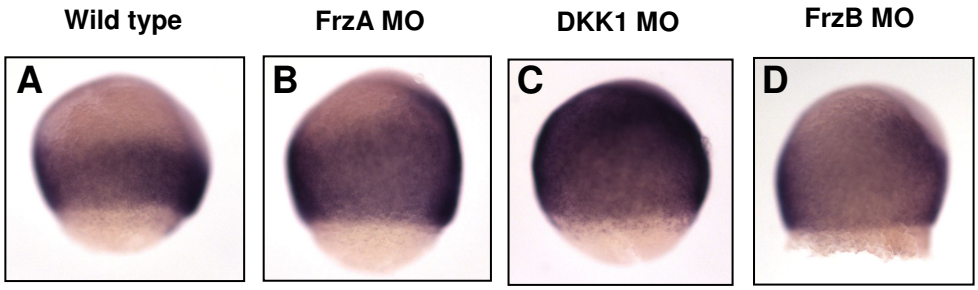


Figure III-22: The inhibition on the Chordin expression by the SFRP1 is specific on the canonical Wnt pathway. The embryo were injected with different morpholino antisense RNA at one cell stage then checked the Axin2 expression at the 75% percent epiboly. All the embryos are lateral view, dorsal part to the right. (A) Wild type, (B) FrzA antisense MO , (C) Dickkopf 1 antisense MO, (D) FrzB antisense MO.

As shown in Fig. III-22, the expression of Axin2 at 75 % epiboly stage is upregulated in all knock-down embryos. The marginal expression of Axin2 in wild type embryo (Fig. III-22A) extends toward the animal pole for FrzA, FrzB and DKK1 knock-down embryos (Fig. III-22B-D). This upregulation of Axin2 was expected for the two Wnt inhibitors FrzB and DKK1. The result observed for FrzA knock-down suggests that this factor is also involved in the regulation of the Wnt signalling.

In addition, FrzA may control the BMP signalling by regulating Chordin expression. Expression of Chordin is under the control of Wnt signalling and this gene is positively controlled by maternal Wnt (β-catenin) and negatively controlled by zygotic Wnt signalling (through the activity of two zygotic Wnt8 target: Vent/Vox which act as chordin transcription inhibitors). Our data show that zygotic Wnt 8 is unlikely to be regulated by FrzA because the downstream marker Vent appeared unaffected by up- or down-regulation of FrzA expression. Similarly, the decrease of Vox expression in FrzA gain of function experiments does not match the reduction of Chordin expression. Since the relationship between FrzA and the Wnt8 signalling is still not clear, I decided to test the direct interaction between the zygotic Wnt8 activity and FrzA.

5. The interaction between FrzA and the zygotic Wnt8:

a. Rescue of Wnt8 overexpression by FrzA RNA co-injection:

In a first experiment I tried to rescue the phenotype resulting from Wnt8 gain of function by FrzA. Wnt8 RNA injection showed that injection of low concentration of Wnt8 RNA can phenocopy the zygotic Wnt8 overexpression phenotype (posteriorization, with anterior brain and eyes deletion, (Kelly et al., 1995) which is observed after injection of a DNA construct expressing Wnt8 under a constitutive promoter. After injection of low dose of Wnt8 RNA alone or

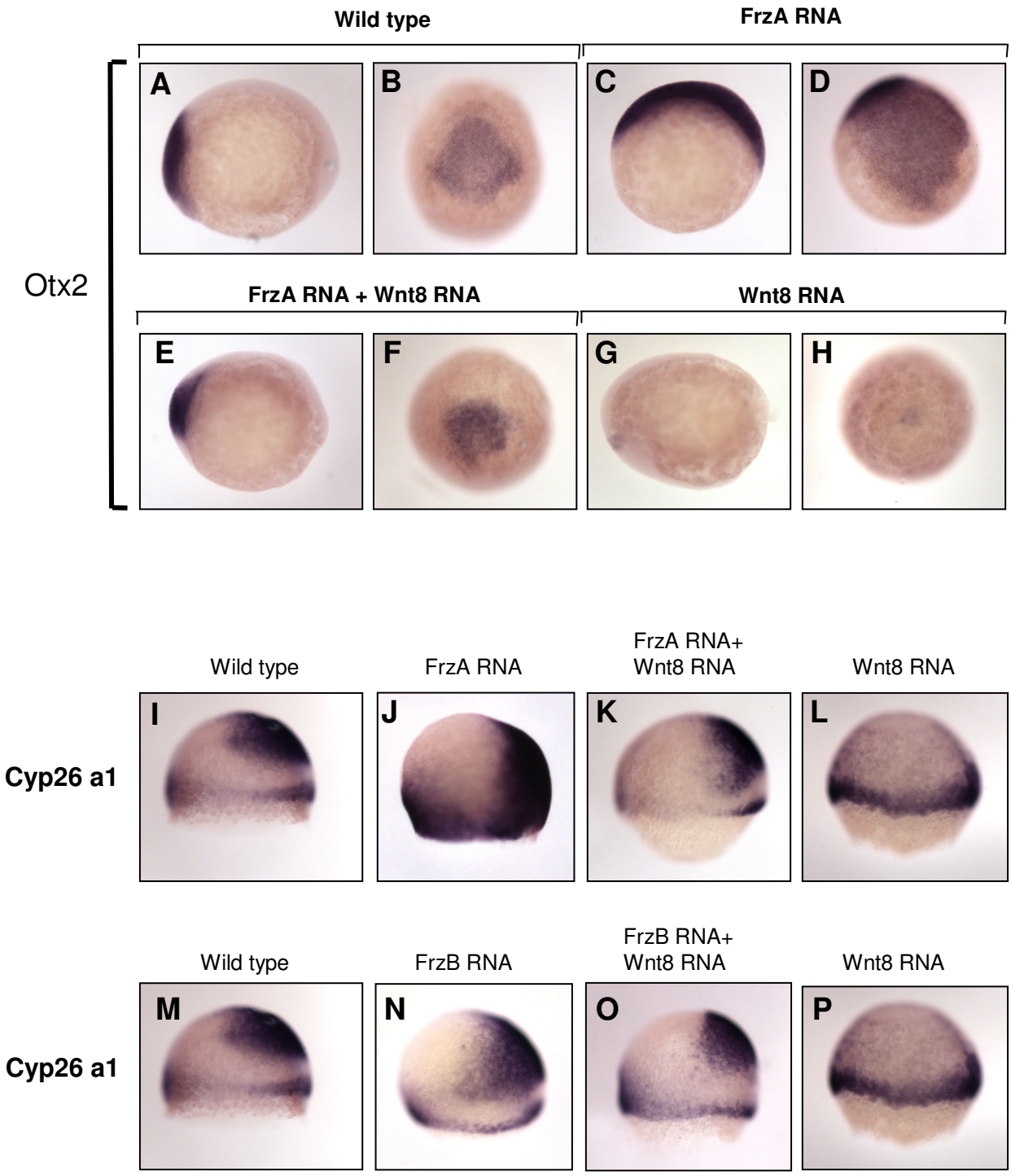


Figure III-23 : The rescue of the Wnt8 overexpression defect by the FrzA RNA overexpression. The embryos were either wild type (A, B, I, M) or injected with FrzA RNA (C, D, J), FrzB RNA (N) or FrzA RNA plus Wnt8 RNA (E, F, K) or FrzB RNA plus Wnt8 RNA (O) or Wnt 8 RNA(G, H, L, P) and developed until tail bud stage (A-H) or 75 % epiboly stage (I- P) and staining with Otx2 (fore brain) marker (A-H) or Cyp26a1 (I-P). The embryo are lateral view and the anterior side to the left and dorsal to the top (A,, C, E, G) or dorsal anterior view and the dorsal to the top (B, D, F, H) or lateral view and the dorsal side to the right and animal pole to the top (I-P).

in combination with FrzA RNA I verified the extension of the posteriorization of the embryo by looking at the expression of two anterior neural plate markers, Cyp26a1 and Otx2. In addition, as a control of a rescue of the zygotic Wnt8 gain of function I performed the same experiments by co-injecting FrzB RNA.

I observed that the loss of the anterior brain resulting from Wnt8 overexpression can be rescued by the FrzA RNA co-injection. The Wnt8 RNA injected embryos loose Otx2 expression (Fig. III-23G, H) as well as Cyp26a1 expression (Fig. III-23L, P) while these two markers were reexpressed again when FrzA RNA was co-injected (Fig. III-23E, F, K). The same observation is made for FrzB RNA co-injection (Fig. III-23O).

This result showed that in these experimental conditions FrzA is able to rescue Wnt8 overexpression defects. This suggests that FrzA can inhibit the Wnt8 signalling but the molecular mechanism of this inhibition is still unknown. Does it act through a direct Wnt8 binding or would it act in parallel?

b. Rescue of FrzA loss of function by Wnt8 knock-down:

If FrzA antagonizes directly Wnt8, then the chordin up-regulation observed in FrzA knock-down embryos may be due to an increase of Wnt8 activity. Therefore I hypothesized that this would be rescued if I decrease Wnt8 activity. I checked this hypothesis by trying to rescue FrzA loss of function phenotype by a progressive knock down of Wnt8 with antisense MO injection.

I injected FrzA antisense MO alone or in combination with different concentrations of Wnt8 antisense MO (from 300-1200 pg) as well as Wnt8 antisense MO alone in embryos at the one cell stage and I analyzed the expression of Chordin at the onset of gastrulation.

Surprisingly, instead of a rescue of the chordin upregulation by lowering the Wnt8 activity I observed an increase of chordin expression (Compare Fig.

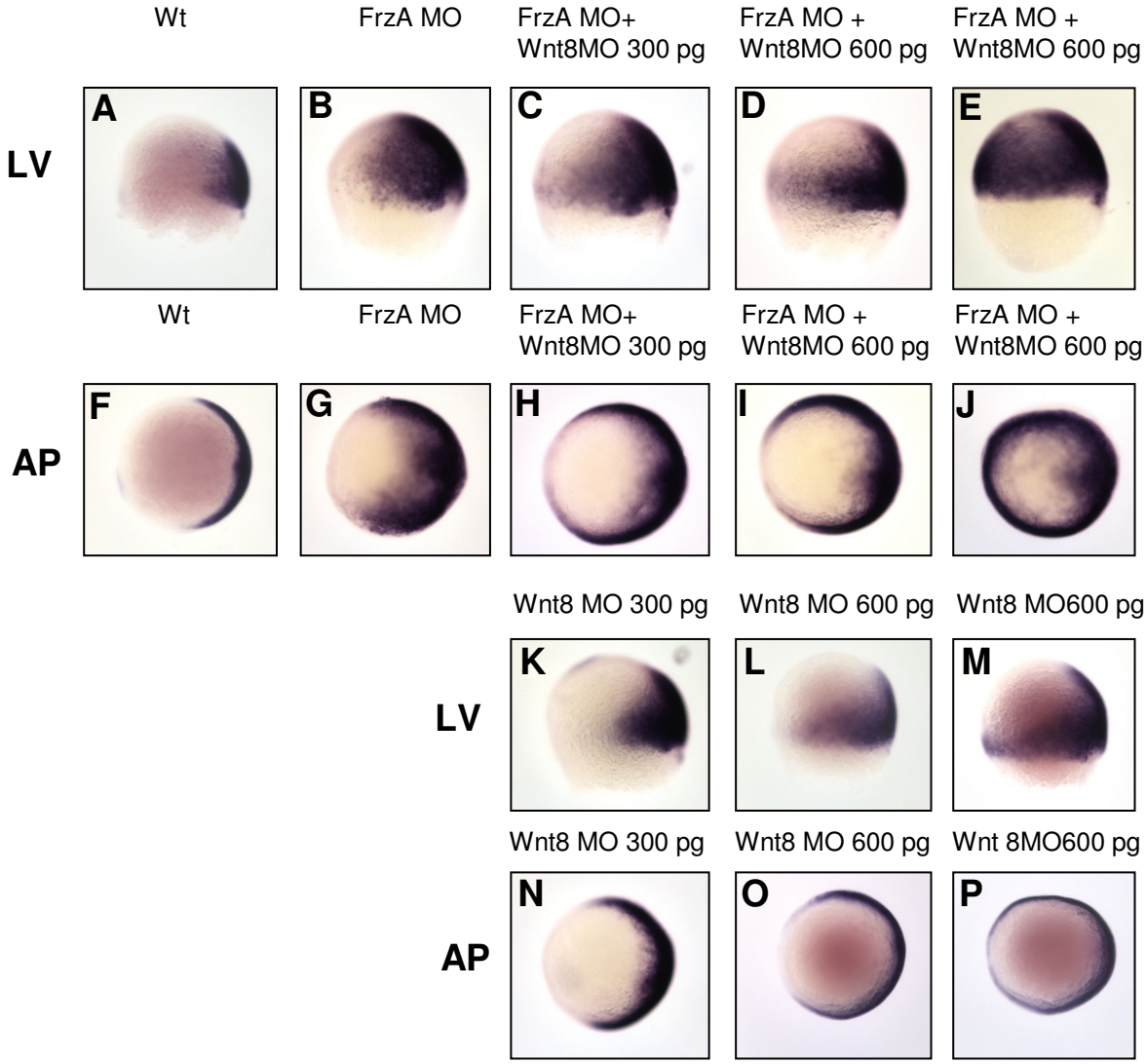


Figure III-24 : Rescue experiment for the FrzA knocked down defect by Wnt8 lost of function. The embryos were either injected with FrzA antisense MO (B, G) or FrzA antisense MO plus different concentration of Wnt8 antisense MO from 300-1200 pg (C-E, H-J, K-M, N-P) or different concentration of Wnt8 antisense MO alone (K-P) and then determined the Chordin expression at shield stage. (A-E, K-M) Lateral view (LV), (F-J, N-P) Animal pole view (AP). All embryos dorsal are to the right.

III-24C-E and B, K-M). This result invalidates the hypothesis that the decreasing Wnt8 expression would rescue the FrzA knock-down phenotype.

c. Overexpression of FrzA in Wnt8 knock-down embryos:

Finally, if FrzA acts through a repression of Wnt8 activity then in a Wnt8 loss of function embryo FrzA should have no effect. To check this hypothesis I co-injected FrzA RNA with Wnt8 antisense MO into the embryo at 1 cell stage then looked for chordin expression at sphere and shield stage. The result showed that FrzA is still able to function and to inhibit chordin expression when the Wnt8 was knocked down (Fig. III-25C and G). In addition to rule out the possibility that the FrzA may participate to the Wnt8 signalling by activating Wnt8 downstream factors like Vent/Vox/Ved that repress chordin expression, I injected different dominant negative form (VP16-Vent, (Onichtchouk et al., 1996)) or antisense MOs (Ved or Vent or Vox) corresponding to these factors with FrzA RNA and I checked the expression of chordin. As shown in figure III-26 for each co-injection combination, the FrzA is able to repress the chordin expression induced by the loss of function of Ved (Ved antisense MO, Fig. III-26B, C), Vent (VP16-Vent RNA, antisense MO, Fig. III-26D-G) or Vox (antisense MO, Fig. III-26H, I).

These results demonstrate that the repression of chordin expression by FrzA occurs without involving chordin transcriptional repressors downstream targets of Wnt8.

d. Summary about interactions between Wnt8 and FrzA:

Taking the above data together, there are several evidences suggesting that FrzA does not function by repressing the Wnt8 signalling pathway:

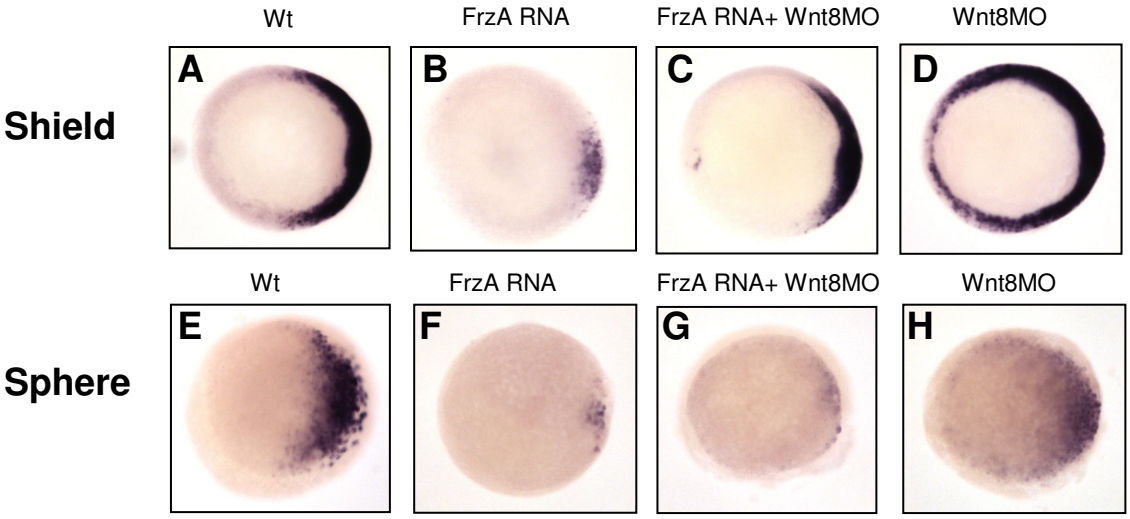


Figure III-25: The inhibition on the canonical Wnt pathway by the SFRP1 is not through the binding to the Wnt8. The embryo were injected with different combination of RNA at one cell stage and checked the Chordin expression at the shield stage (A-D) or at the sphere stage (E-H). All the picture are the animal pole view. The combination are (A, E) Wild type, (B,F) FrzA RNA, (C, G) FrzA RNA+ Wnt8 antisense MO, (D,H) Wnt8 antisense MO.

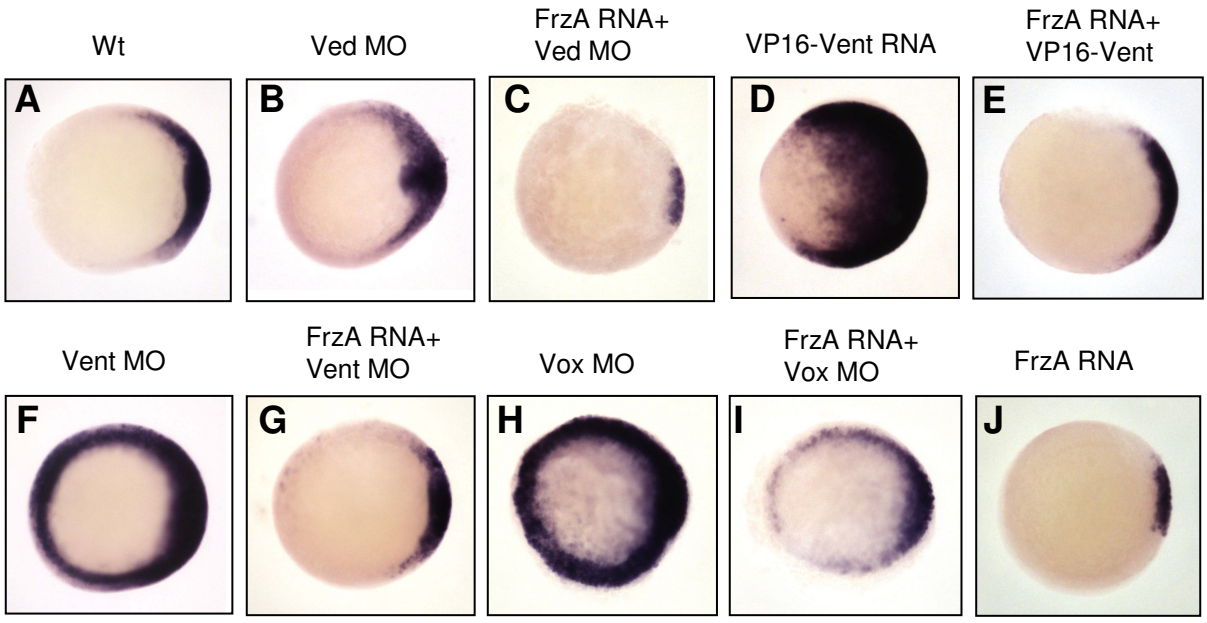


Figure III-26: The inhibition on the canonical Wnt pathway by the SFRP1 is not through inhibiting the zygotic Wnt8 pathway. The embryo were injected with different combination of RNA at one cell stage and checked the Chordin expression at the shield stage. All the picture are the animal pole view and dorsal to the right side. The combination are (A) Wild type, (B) Ved antisense MO, (C) FrzA RNA+ Ved antisense MO, (D) VP16-Vent RNA, (E) FrzA RNA+ VP16-Vent RNA, (F) Vent antisense MO, (G) FrzA RNA+ Vent antisense MO, (H) Vox antisense MO, (I) FrzA RNA+ Vox antisense MO, (J) FrzA RNA.

(1) Inhibition of either Wnt8 or Wnt8 downstream factors cannot totally inhibit the ability of FrzA to repress chordin expression.

(2) Comparison of the different markers used (Cyp26a1, chordin, and Vent) showed that FrzA gain of function affects the embryo differently than a Wnt8 loss of function.

(3) Chordin and Cyp26a1 expressions territories differ in FrzA and FrzB or DKK1 overexpression conditions.

Nevertheless there are also some lines of evidence suggesting that FrzA may affect the Wnt signalling pathway:

(1) FrzA overexpression can rescue the Wnt8 overexpression posteriorization defects.

(2) Up-regulation of Axin2 in the FrzA knocked down embryo may indicates the Wnt signalling is up-regulated and possibility the same as inhibiting the other Wnt signalling antagonists FrzB and DKK1.

Therefore, there are some contradictory results between the data issued from FrzA/Wnt8 co-overexpression and data coming from FrzA overexpression in Wnt8 signalling knock-down context. However, because of the evidences showing that FrzA activity is not related to Wnt8 activity, I hypothesized that FrzA may act in parallel of Wnt8 to inhibit some other aspects of the Wnt signalling.

6. Mechanism of action of FrzA for the repression of chordin:

a. FrzA act at short range

I tried to determine the range of action of FrzA in order to identify the possible mechanisms of its control on Chordin expression. To do this, I

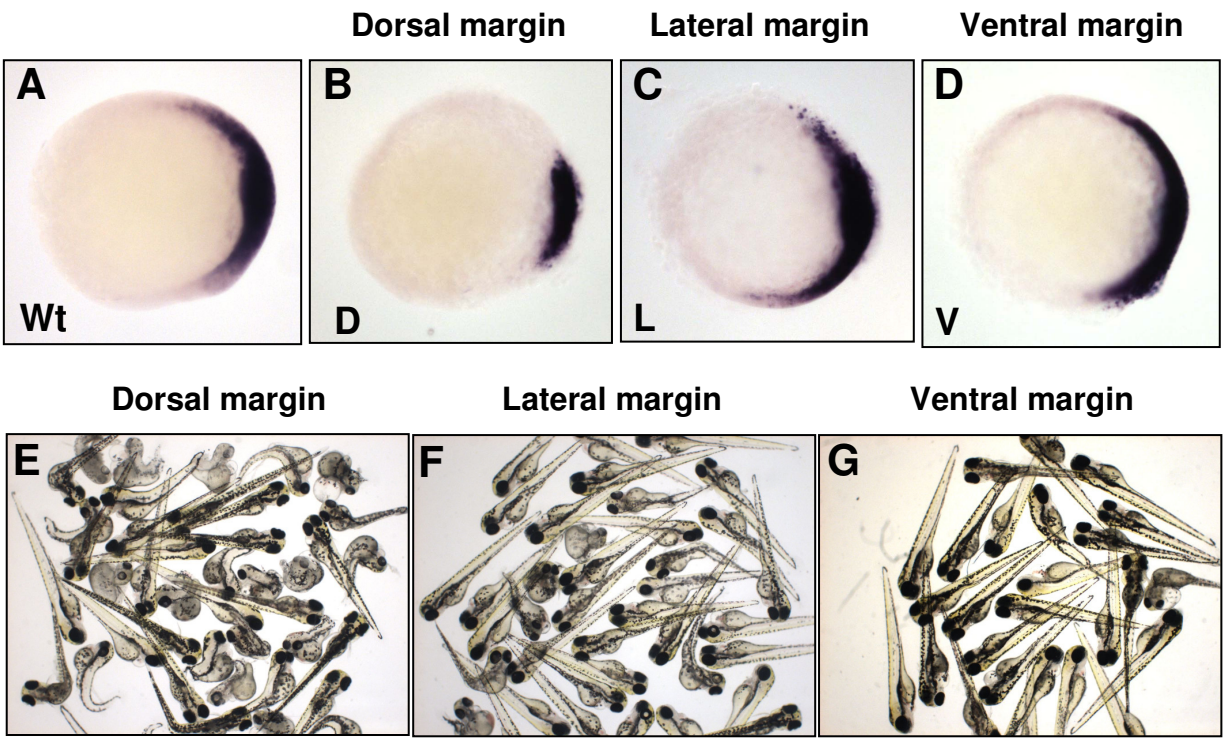


Figure III-27 : The effect of local FrzA overexpression on the chordin expression and the phenotype. (A-D) The chordin expression or (E-G) 72 hrs phenotype after the local FrzA overexpression. Wt: wild type, different positions of FrzA overexpression are labeled above each picture. All embryos in (A-D) are in the animal pole view, dorsal part to the right. Only the Dorsal FrzA overexpression can decrease the Chordin expression and have high percentages of influence on the phenotype.

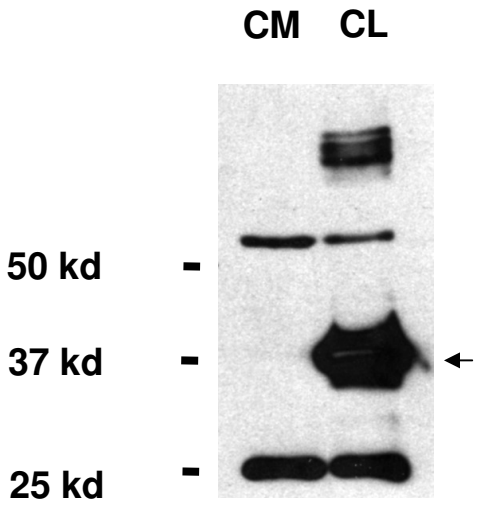


Figure III-28 :The expression region of FrzA in the cell culture system. The FrzA flag-tag plasmid was transfect into the 293T cell line and after take conditional medium (CM) or cell lyses (CL) to make the immunoprecipitation with anti flag beads and also do the western blot with anti-flag antibody. The arrow shows the FrzA expression.

injected FrzA RNA locally at the 16 cell stage and I checked for the position of the corresponding clones at the onset of gastrulation. Then I separated embryos based on the position of clones at the dorsal, lateral and ventral margin. I separated the embryos into two batches one for which I checked the expression of chordin (Fig. III-27B-D) and the second one for which I let the embryos develop until late developmental stages. Surprisingly expression of chordin decreased only when the clones were present at the dorsal margin. Similarly, only the embryos deriving from a dorsal clone displayed a strong phenotype (Fig. III-27B) while embryos derived from lateral or ventral clones appeared wild type (Fig. III-27E-G). Because ventral and lateral clones expressing FrzA are unable to affect chordin expression this suggests that FrzA can only act at short range.

To further confirm this point, I also used a cell culture system to detect the FrzA secretion. I tagged the FrzA protein with a flag domain in C-terminal which has no influence on the protein activity and I transfected this construct into Human 293T cell line. After immunoprecipitation using either the culture medium or a cell extract I visualized the presence of the FrzA-flag protein by western blot with an anti-flag antibody. As shown in Figure III-28, FrzA cannot be detected in the conditional medium and is only observed in the cell lysis. This result is consistent with a previous report on human and mouse sFRP1 that shows this protein to be associated with the extracellular matrix or present in the cells but absent in the culture medium (Bafico et al., 1999).

These data suggest that after secretion FrzA remains associated with the cell extracellular matrix (ECM) and functions only in a paracrine mode as it has been suggested previously (Bafico et al., 1999). This is likely why in the embryo FrzA acts at a short range as shown by the effect of my local injection.

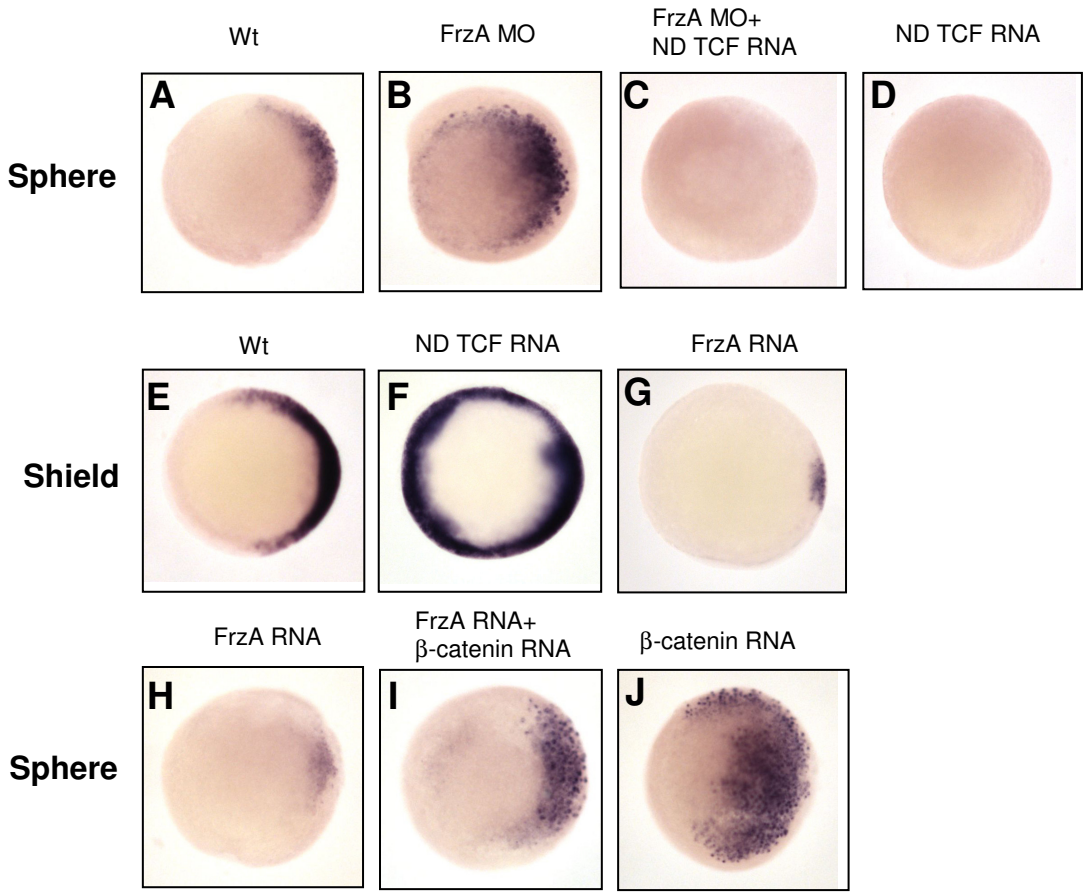


Figure III-29 : The inhibition on the Chordin expression by the SFRP1 is specific on the canonical Wnt pathway. The embryos were injected at on cell stage with different combinations then check the Chordin expression at sphere (A-D, H-J) or shield (E-G) stage. The combinations are (A,E) wild type, (B) FrzA antisense MO, (C) FrzA antisense MO+ N-terminal deletion (ND) TCF RNA, (D, F) N-terminal deletion (ND) TCF RNA, (G, H) FrzA RNA, (I) FrzA RNA+ β-catenin RNA, (J) β-catenin RNA. All the embryos are animal pole view and dorsal part to the right.

Based on these results and as FrzA and the zygotic Wnts are expressed in complementary territories I made the hypothesis that FrzA inhibits chordin expression by negatively interacting with the maternal Wnt signalling.

b. FrzA inhibits Chordin expression by interfering with the maternal Wnt activity.

In order to investigate this hypothesis I attempted to modify maternal Wnt signalling activity in the embryo. First I used a constitutively active form of TCF3. TCF3 is a transcriptional repressor which blocks Wnt target genes expression. Upon stimulation by Wnt, the β-catenin is translocated to the nucleus, interacts with TCF3 and prevents it from continuing to inhibit the Wnt target genes. The TCF constitutively activated form is a truncated molecule lacking the N-terminal domain for TCF3 (ND-TCF3) that is known to interact with the β-catenin. Injection of ND-TCF3 RNA in the embryo results in a complete loss of chordin expression at the sphere stage, indicative of a complete inhibition of maternal Wnt activity. Conversely, when the chordin expression was analyzed at the shield stage, I found a strong expansion of chordin expression in lateral and ventral domains (Fig. III-29F), indicating of an inhibition of zygotic Wnt activity.

MO knocked down of FrzA results in a strong induction of chordin at the sphere stage (Fig. III-29B) but in injections of both antisense MO-FrzA and ND-TCF3 chordin expression is repressed (Fig. III-29C). Therefore the constitutive repression of chordin transcription by TCF3 is epistatic on the induction of chordin expression in FrzA loss of function. Altogether, these data shows that FrzA may participate in the regulation of maternal Wnt signalling as its absence upregulates this pathway and this upregulation can be specifically blocked by a repressor of maternal Wnt activity.

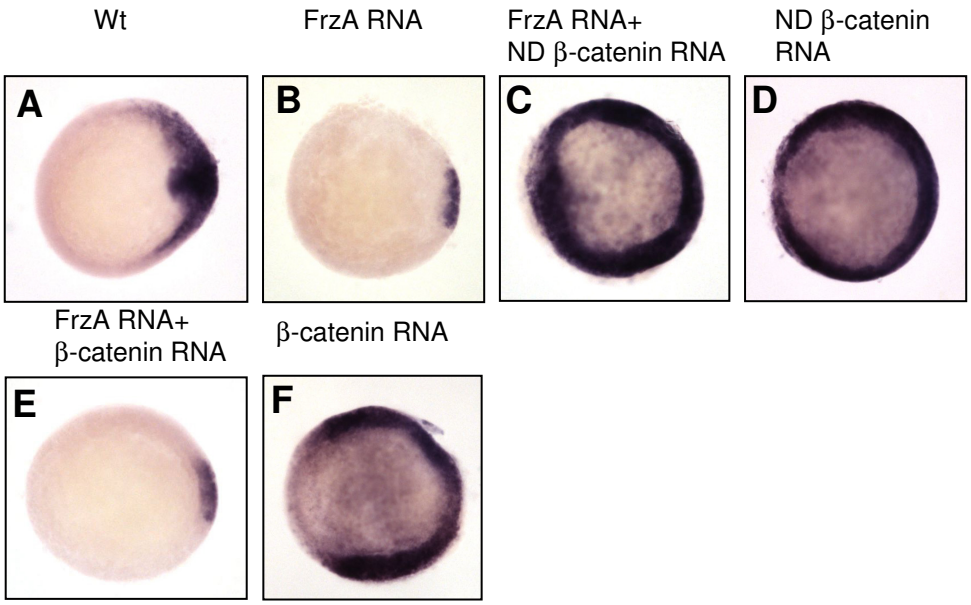


Figure III-30: The inhibition on the Chordin expression by the SFRP1 is specific on the canonical Wnt pathway. There are different combination of RNA injection at one cell stage and check the chordin expression at the shield stage. (A) wild type, (B) FrzA RNA, (C) FrzA RNA+ N-terminal deletion β-catenin (ND β-catenin) RNA, (D) ND β-catenin RNA (E) FrzA RNA+ β-catenin RNA, (F) β-catenin RNA. All the embryos are animal pole view and dorsal to the right. FrzA overexpression can reduce the increased β-catenin signaling from full length β-catenin but not ND β-catenin.

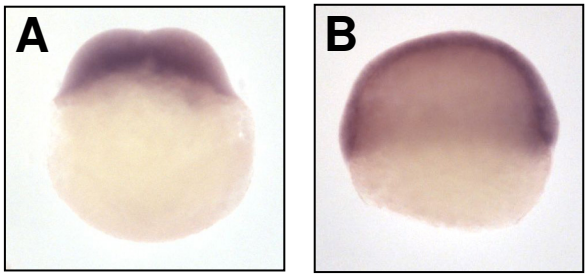


Figure III-31: The expression of Frizzled 2 receptor (Fzd2) transcripts in the zebrafish early development. The Fzd2 transcripts were detected by *in situ* hybridization at 2 cells (A) or shield (B) stage.

In a second approach I used the β-catenin which is the main intermediate of canonical Wnt pathway and whose overexpression can induce the expression of maternal Wnt target genes (Kelly et al., 2000). After injection of β -catenin RNA I checked the chordin expression at sphere stage and observed a strong induction of its expression (Fig. III-29J). Coinjection of FrzA can decrease induction of Chordin expression by the β-catenin (Fig. III-29I). This clearly indicates that FrzA interferes with the canonical Wnt signalling pathway by regulating the ability of β-catenin to induce Chordin expression.

The regulation of the β-catenin activity is such that in absence of stimulation by Wnt, the N-terminal region of β -catenin is phosphorylated by the glycogen synthase kinase 3β (GSK3 beta, (Bienz, 2005)) and this phosphorylation eventually results in its degradation. In consequence an N-terminal deletion form of β-catenin that cannot be phosphorylated cannot be degraded. Therefore this truncated form is constitutively activated and induces canonical Wnt target genes (Ryu et al., 2001).

Both the full length and N-terminal deletion of β-catenin can induce chordin expression (Fig. III-30D, F) as it has been previously reported (Wessely et al., 2001). Nevertheless when FrzA RNA is coinjected, the extension of chordin expression induced by the full length β-catenin can be reduced (Fig. III-30E) but this is not the case for the induction generated upon injection of the N-terminal deletion of β-catenin (Fig. III-30C).

This result suggests that the reduction of Chordin expression due to the overexpression of FrzA results from degradation of the β-catenin and therefore form an interaction with the canonical Wnt pathway and by a parallel mechanism resulting in the induction of other chordin transcriptional repressor(s).

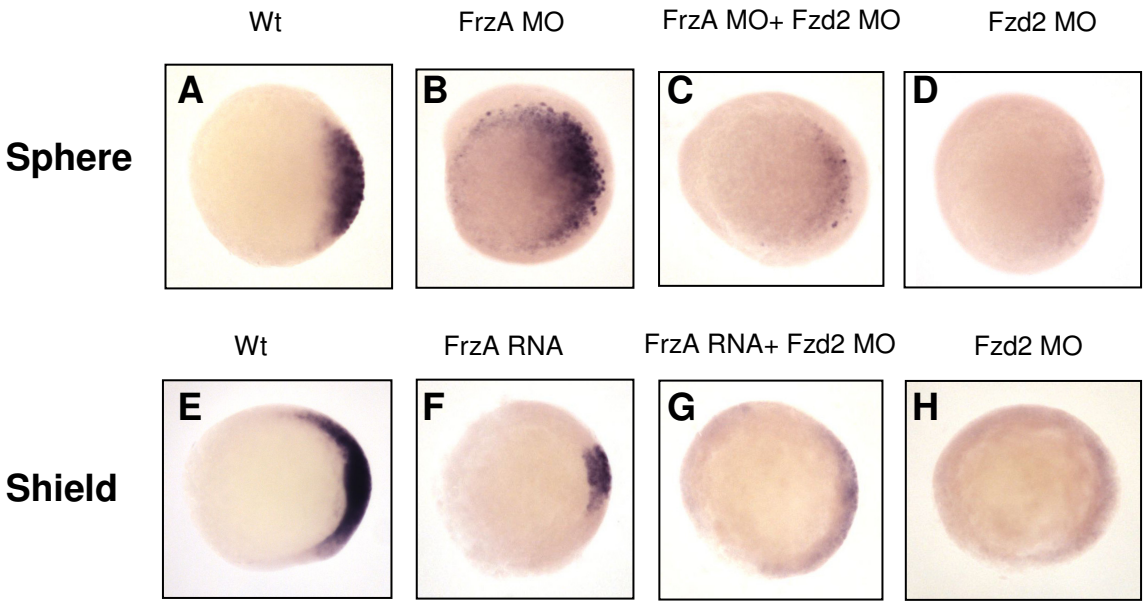


Figure III-32: The inhibition of the canonical Wnt pathway by the SFRP1 may through the Fzd2. All the embryos were injected with different combination of RNA at one cell stage then check the Chordin expression at sphere (A-D) or Shield (E-H) stage, all the embryos are from the animal pole view. The combinations are (A) Wild type, (B) FrzA MO, (C) FrzA MO+ Fzd2 MO, (D) Fzd2 MO, (E) Wild type, (F) FrzA RNA, (G) FrzA RNA+ Fzd2 MO, (H) Fzd2 MO.

In addition, this results indicates that FrzA acts upstream of the phosphorylation of the β-catenin to regulate the maternal Wnt activity.

Previous studies on SFRP1 revealed the ability of this factor to inhibit Wnt1, Wnt3a, Wnt7, Wnt8 and *Drosophila* Wingless (Wg) activity in cell culture using a TCF dependent reporter assay or embryo co-injection assays (Bafico et al., 1999; Dennis et al., 1999; Finch et al., 1997; Uren et al., 2000; Xu et al., 1998). In addition, co-immunoprecipitation results of SFRP1 with Wnt1, Wnt2 or Wg have also been reported. These results suggest that FrzA may inhibit the Wnt signalling through binding to Wnt molecules and prevent them binding to Frizzled receptor. However, there are also evidences suggesting that SFRP1 can directly bind to the Frizzled receptor (Bafico et al., 1999) and affects Wnt signalling without binding to the Wnt ligands (Rodriguez et al., 2005).

7. FrzA acts at the level of the Frizzled 2 receptor (Fzd2):

a. The effect of up-or down-regulation of FrzA expression is possibly through Fzd2.

According to a previous report (Rodriguez et al., 2005) SFRP1 can directly bind to Frizzled 2 receptor. Therefore I checked the expression of Fzd2 during early developmental stages by *in situ* hybridization. The Frizzled 2 receptor is both maternally and zygotic expressed and its RNA is uniformly distributed in the embryo before gastrula stage (Fig. III-31A, B).

I performed Fzd2 loss of function experiments by antisense MO knock down first then with additional up- or down-regulation of FrzA and followed by the analysis of Chordin expression at the sphere or the shield stages. In absence of Fzd2, chordin expression is not induced in the embryo and the phenotype is epistatic on FrzA loss of function phenotype (Compare Fig.

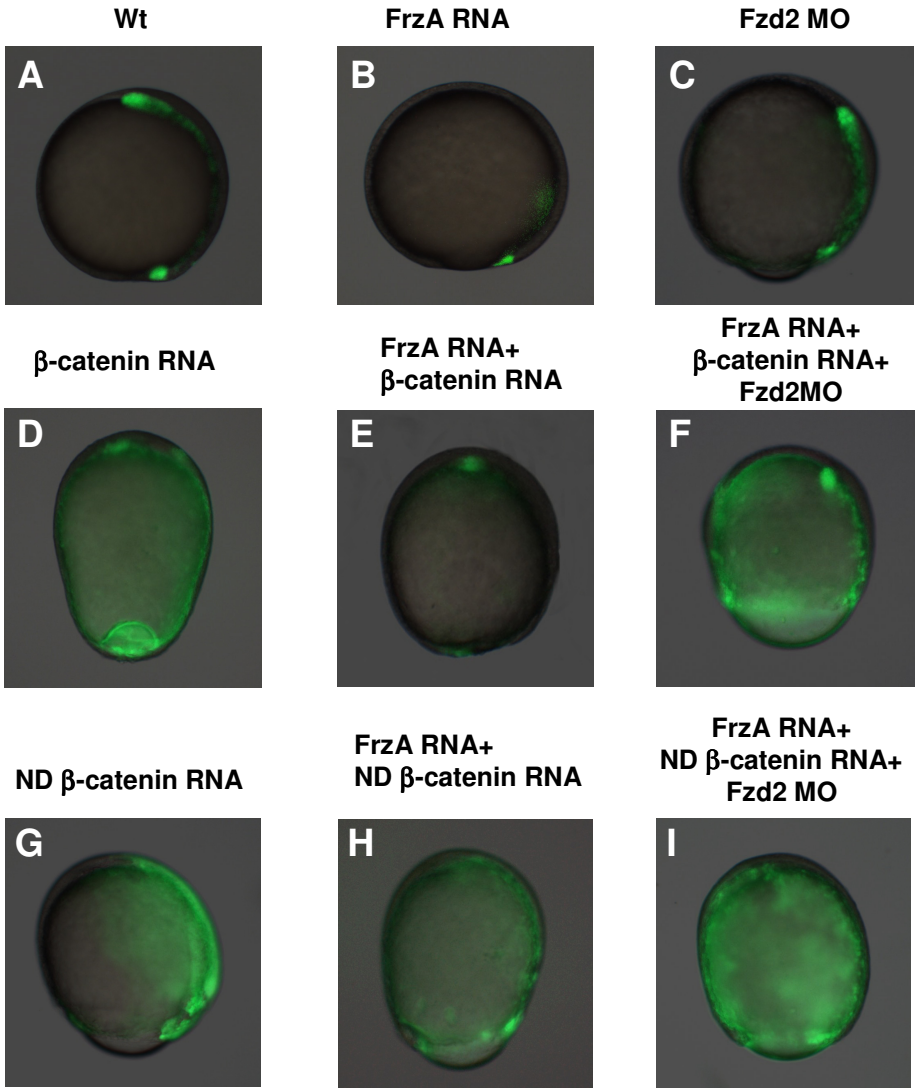


Figure III-33: The inhibition of the canonical Wnt pathway by the SFRP1 may through the Fzd2. All the embryos were injected with different combination of RNA at one cell stage in the Dharma-GFP transgenic fish and check the GFP expression at tail bud stage. The combinations are (A) Wild type, (B) FrzA RNA, (C) Fzd2 antisense MO, (D) β-catenin RNA, (E) FrzA RNA+ β-catenin RNA, (F) FrzA RNA+ β-catenin RNA+ Fzd2 antisense MO, (G) N-terminal deletion (ND) β-catenin RNA, (H) FrzA RNA +ND β-catenin RNA, (I) FrzA RNA+ β-catenin RNA+ Fzd2 antisense MO. All the embryos are lateral view and dorsal to the right.

III-32B, C). These datas demonstrate that the Frizzled 2 receptor acts downstream of FrzA and may be a main mediator for FrzA activity. However, I still need a direct evidence to show that loss of function of Fzd2 receptor will block the FrzA signalling.

b. The Fzd2 receptor mediates the FrzA inhibition signalling on the maternal β-catenin pathway.

To further test whether FrzA acts through the Frizzled 2 receptor, I investigated the role of Frizzled 2 receptor on the FrzA inhibition of β-catenin activity. I already showed that FrzA can inhibit Chordin expression resulting from injection of full length β-catenin RNA but not the induction of Chordin by the ND β-catenin RNA overexpression.

In addition to the analysis of Fzd2 activity at the level of chordin expression, I also used Dharma-EGFP transgenic fish that I generated to study the role of this receptor in the inhibition of β-catenin signalling by FrzA.

Dharma (also named Bozozok) is a transcription factor expressed very early at the dorsal margin and its promoter contains a Tcf/Lef binding site. This gene has been shown to be regulated by the maternal β-catenin signalling (Ryu et al., 2001). I used the Dharma promoter fused with EGFP (constructed provided by Hibi's lab). In transgenic embryos the expression pattern of the EGFP under the control of this promoter is the same than the expression pattern of Dharma established by *in situ* hybridization (but the EGFP labelling stays longer in the tissue due to the high stability of this protein).

To investigate the role of Fzd2 on the FrzA function I injected FrzA RNA alone, Fzd2 antisense MO alone, β-catenin alone, β-catenin plus FrzA RNA, β-catenin plus FrzA RNA plus Fzd2 antisense MO. As a negative control for FrzA inhibition on the β-catenin signalling, I also use the ND

β-catenin alone, ND β-catenin plus FrzA RNA, ND β-catenin plus FrzA RNA plus Fzd2 antisense MO to compare those results with those obtain with the full length β -catenin.

In these experiments I observed that expression of EGFP is decreased in FrzA RNA injected embryos (Fig. III-33B) but strongly increased in both β-catenin or ND β-catenin injected embryos (Fig. III-33D, G). These observations are similar to those made on the effect of these factors on the expression of Chordin. However, while Fzd2 loss of function abolishes Chordin expression, the injection of Fzd2 antisense MO alone has no effect on EGFP expression (Fig. III-33C).

After co-injection of Fzd2 antisense MO and FrzA RNA I observed that FrzA cannot inhibit β-catenin signalling anymore. This result clearly indicates that Fzd2 is mediating the FrzA function.

Finally I analyzed the effect of FrzA on the phenotype resulting from Fzd2 overexpression. Injection of Fzd2 RNA in the whole embryo at one cell stage results in the induction of Chordin all around the margin. A local injection in one cell at the 64 cells stage results in the ectopic expression of Chordin when the corresponding clone is on the lateral or ventral side (Fig. III-34E-G) but not when it is located at the animal pole (Fig. III-34D) in a domain in which FrzA is expressed.

By double injection of Fzd2 and FrzA RNA I showed that the ectopic induction of chordin expression by Fzd2 overexpression is inhibited by FrzA (Fig. III-34C). There are several different mechanisms that could explain the induction of chordin after Fzd2 overexpression. This might result from the formation of Fzd2 homodimer that will activate the maternal Wnt pathway as it has been previously shown in Xenopus for Xfzd3 (Carron et al., 2003).

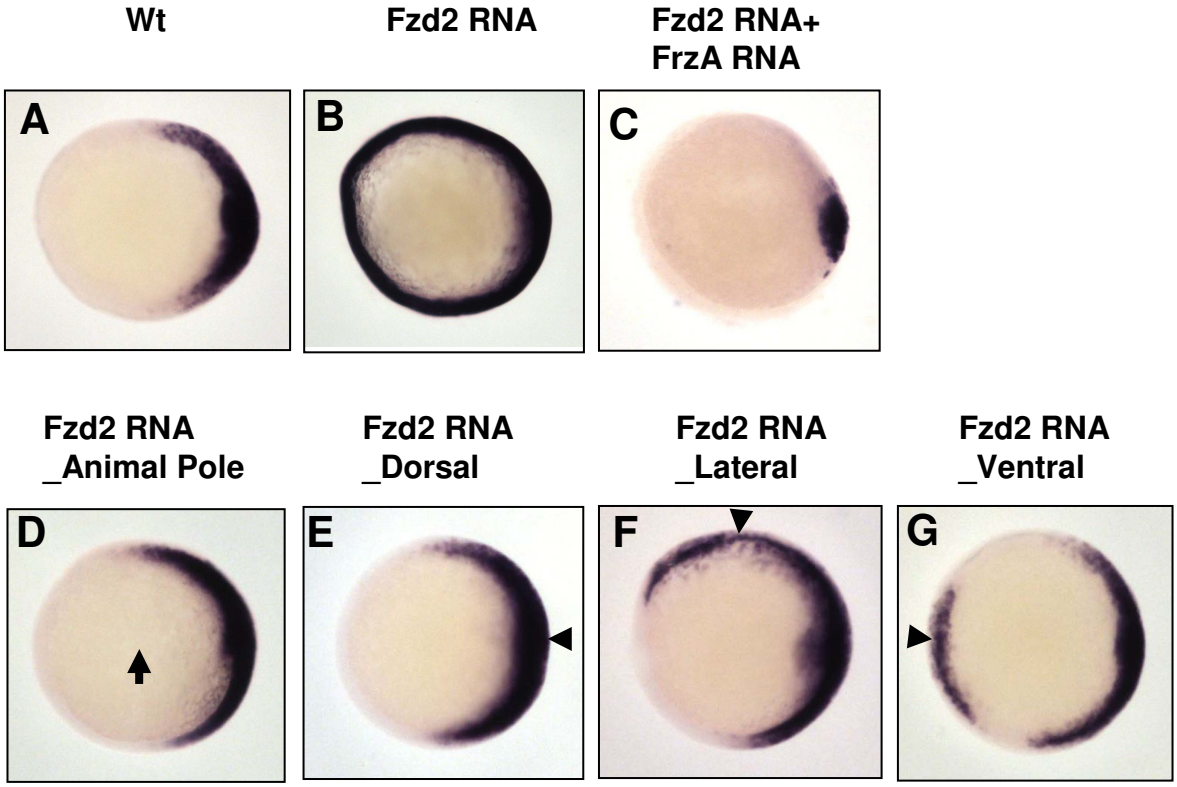


Figure III-34 : The effect of local or overall overexpression of Fzd2 on the chordin expression. The one cell stage embryos were injected with Fzd2 RNA alone (B) or Fzd2 RNA plus FrzA RNA (B) and checked the chordin expression at shield stage. (D-G) The 64 cells stage embryo injected with Fzd2 RNA locally on the Animal pole (D) or Dorsal margin (E) or lateral margin (F) or ventral margin (G) then also checked chordin expression at shield stage. It showed either the whole embryo or local injection, the chordin expression can be induced by Fzd2 overexpression on the margin but not animal pole. The arrow heads indicate local injection position. All embryos are in the animal pole view, dorsal part to the right.

Alternatively it may also result from an increase of the intracellular calcium concentration that will inhibit the zygotic Wnt8 pathway.

Whatever mechanism is involved that would explain the activation of Chordin expression in lateral and ventral marginal domain after injection of Fzd2 I show that FrzA is able to block this function. However, from our previous experiment it is likely that FrzA inhibits the β-catenin stabilisation by directly binding to Fzd2 and blocking its activity. Interestingly, for Xfzd3 dimerization has been shown to require intramolecular and/or intermolecular disulfide linkages, and the N-terminal extracellular region of the receptor, including the cysteine-rich domain (CRD), have been shown to be sufficient for dimerization. Because FrzA contains also CRD motifs it may bind to Fzd2 and prevent its dimerisation explaining the inhibitory effect on downstream events, especially stabilization of the β-catenin, of the maternal Wnt signalling pathway.

c. The FrzA can interact with Wnt 8, Wnt11 and Fzd2.

From the data above we already know that FrzA may function without interfering Wnt8 signalling and that it can inhibit the maternal β-catenin signalling likely through the Fzd2. However, the direct protein-protein interaction between FrzA and Fzd2 has to be demonstrated. .

To determine the interaction between FrzA and Wnts or Frizzled receptors, I chose to use Wnt8 and Wnt11 as canonical and non canonical Wnt representatives, as well as the Fzd2. As a negative control I also used the transcription factor ladybird1 (Jagla et al., 1997). I tagged FrzA with a Flag domain at its C-terminal end and Wnt8, Wnt11, Fzd2 and Ladybird with a Myc domain also placed in the C-terminal region. The effect of this modification has been assessed in vivo by injecting RNA coding for these tagged proteins and I

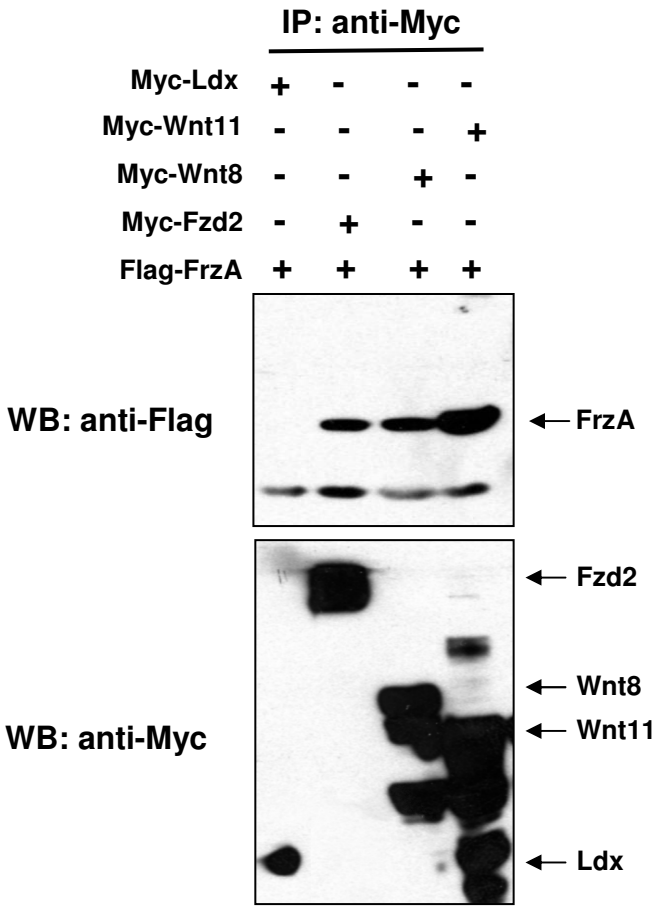


Figure III-35: Interaction between FrzA and Wnt8, 11 and Fzd2.
293T cells were co-transfected with Flag-tag FrzA with Myc-tag Wnt8 or Wnt11 or Fzd2 or Ladybird (Ldx) which is a cytosolic protein. Transfected cells lysates were then subjected to immunoprecipitate (IP) with anti-Myc antibody and then detected by either anti-Myc (bottom) or anti-flag (top) antibody by western blot. After western blot, data from the FrzA-flag detection indicates FrzA can bind to Wnt8 or Wnt11 or Fzd2 but not Ldx.

found that the phenotypes generated at 24hpf were identical to phenotypes obtained upon injection of RNA encoding unmodified proteins. Then I cotransfected in 293T cells, constructs of FrzA-Flag with Wnt8-Myc, Wnt11-Myc, Fzd2-Myc or Ladybird-Myc. Lysates of transfected cells were subjected to immunoprecipitation (IP) using an anti-Myc antibody conjugated with agarose beads and finally the presence of FrzA in the precipitate was detected on Western blot using an anti-Flag antibody.

As shown on Fig. III-35, FrzA can be immunoprecipitated with Wnt8, Wnt11 and Fzd2 but not with the Ladybird protein. FrzA, Wnt8, Wnt11 and Frizzled have a cysteine-rich domain region (CRD), known to be involved in the interaction between Wnts and Frz and it is very likely that the CRD region of FrzA is able to bind to the CRD region of Wnt8/11 or Fzd2. This is in good agreement with a previous report showing that the CRD region of the human sFRP1 can bind to the CRD region of Wnt1, Wnt2 and Frizzled 6 receptor (Bafico et al., 1999).

The data from co-immunoprecipation experiments suggest that FrzA can bind to different CRD containing molecules including Wnt8, Wnt11 and Fzd2. Based on my previous experiments FrzA is most likely to bind to Fzd2 and to inhibit the β-catenin signalling. A previous work on the human sFRP1 suggests that it can bind to Frizzled 6 receptor and may form a non-functional complex (Bafico et al., 1999). Therefore I made the hypothesis that at early developmental stages FrzA will bind and form a non-functional complex with Fzd2. If this is true, then the loss of function phenotype of Fzd2 should be identical to the gain of function phenotype of FrzA.

d. Comparison of Fzd2 loss-of-function and FrzA gain of function

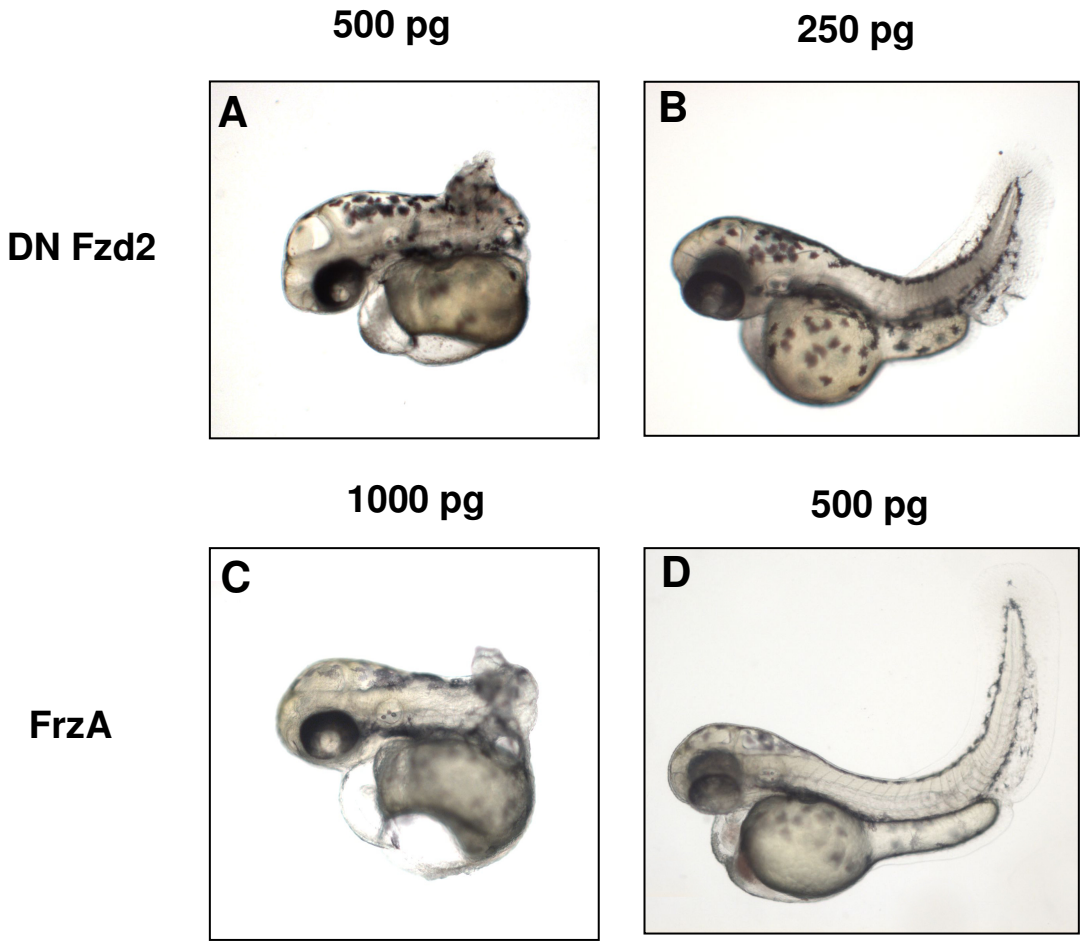


Figure III-36 : The 24 hours phenotype of Dominant negative Frizzled2 receptor RNA (DN Fzd2) or FrzA RNA injection embryo. The embryos were injected with different concentration (labeled on top) of DN Fzd2 RNA (A, B) or FrzA RNA (C, D) at one cell stage and checked the phenotype at 24 hours. In more series phenotype, both DN Fzd2 and FrzA RNA injected embryos show truncated trunk but more relatively intact head (A, C). In the mild affect phenotype, both DN Fzd2 and FrzA RNA injected embryos show upward curled tail which lacks posterior notochord and mild cyclopia.

As showed before, antisense MO knock down of Fzd2 completely remove chordin expression at early stage, a phenotype stronger than the phenotype of FrzA overexpression (compare Fig. 36F, G). This suggests that FrzA/Fzd2 complex may still have a weak basal level of activity on maternal Wnt signalling. Alternatively, FrzA protein produced after RNA injection may be unable to bind and completely inhibit all Fzd2 receptors. Finally, FrzA is likely to compete with the endogenous Wnts for binding to the Fzd2 making difficult to get a complete inhibition. Therefore I tried to perform a partial knock-down of Fzd2 to see if this can mimic the effect of FrzA overexpression.

To do so, I generated Fzd2 forms lacking the C-terminal domain from the motif KTxxxW which has been shown to be the major binding site for the PDZ domain of Dishevelled and which is required to recruit Dishevelled to the membrane, a step essential to activate the canonical Wnt pathway (Cong et al., 2004; Umbhauer et al., 2000).

Embryos were injected with this dominant negative form of Fzd2 at the one cell stage and then checked their phenotype at 24 hours and compare this phenotype to 24 hours FrzA RNA injected embryos phenotype. When injecting high concentration of either DN-Fzd2 or FrzA RNA embryos show very similar phenotype. This is characterized by a posterior truncation of the embryo which appears mainly unaffected in head and anterior trunk (Fig. III-36A, C). For a moderate amount of RNA injected, embryos show an upward curled tail which lacks posterior notochord and mild cyclopia.

However, while morphologically overexpression of FrzA and inhibition of Fzd2 are similar, their effects on the embryo patterning are different. I compared FrzA overexpressing embryos with embryos for which Fzd2 activity was knocked down either by injection of RNA coding for DN-Fzd2 or by

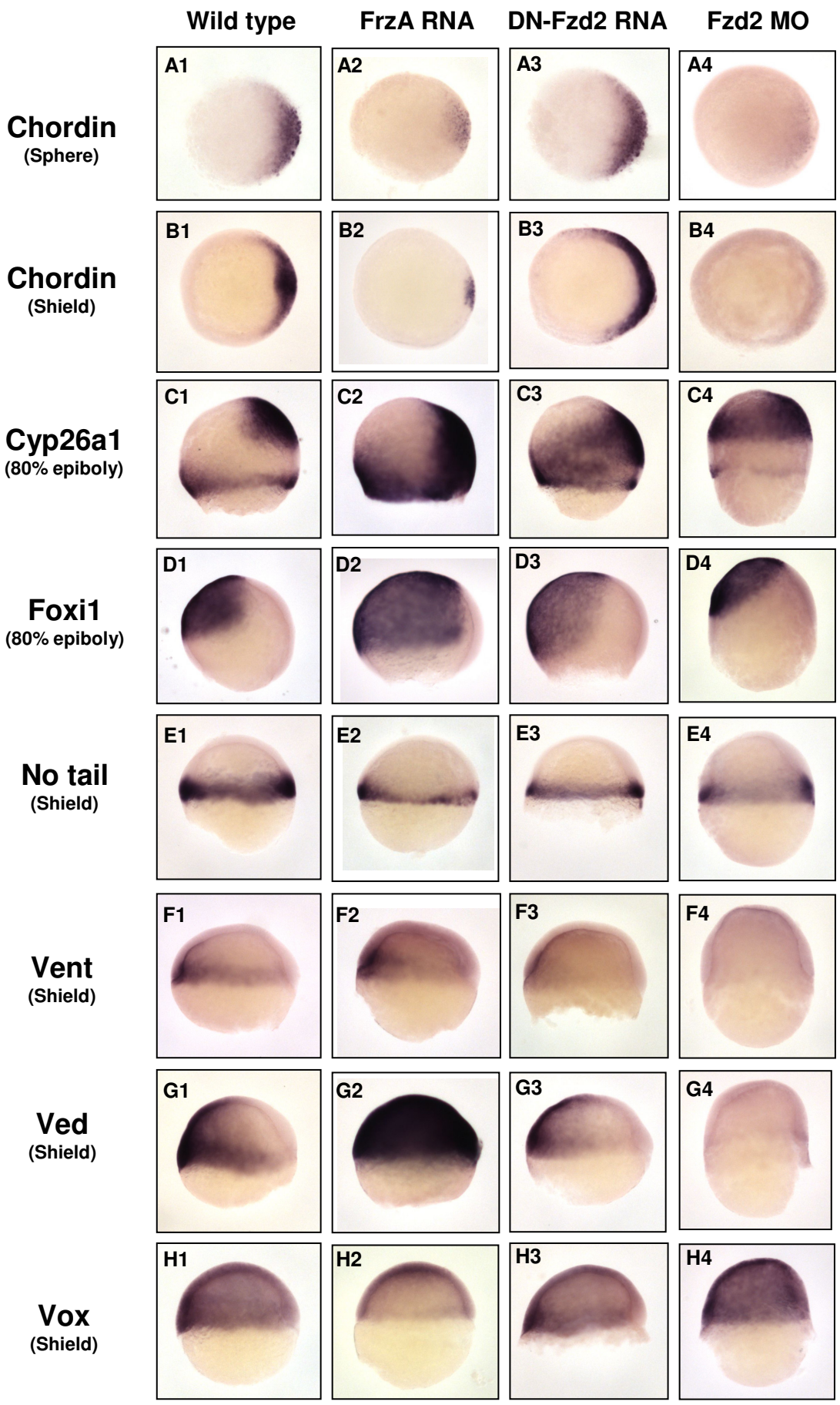


Figure III-37: Comparison of different markers expression upon FrzA overexpression or Fzd2 inhibition. Different column indicates wild type, FrzA overexpression, Fzd2 inhibition by C-terminal truncation from RNA (DN-Fzd2) or antisense MO injection. The markers are labeled on the left of each row and all the embryo are lateral view and dorsal to the right except A1-A4, B1-B4 are animal pole view.

injection of antisense MO. The markers I choose for my analysis were chordin, Cyp26a1, Foxi1, No tail, ved, vent and vox.

I observed that embryos injected with Fzd2 antisense MO lost chordin expression at the sphere stage. This is not the case for DN-Fzd2 injected embryos at sphere and shield stage (compare III-37A1-A4, B1-B4). Fzd2 loss-of-function results in embryos displaying an anteriorization of the neural plate characterized by an extension of Cyp26a1 expression in presumptive hindbrain and spinal chord territories (Fig. III-37 C3, C4) while FrzA overexpression embryos displays an extension of the dorsal expression of Cyp26a1 toward the margin (Fig. III-37C2). Analysis of Foxi1 expression, a marker of epidermis, allows visualizing the difference of phenotypes between FrzA overexpression and Fzd2 loss of function (Fig. III-37D2-D4). In addition, marginal extension of ectodermal markers correlates with a decrease of the expression of the panmesodermal marker no tail in FrzA overexpressing embryos but not Fzd2 antisense MO injected embryos (Compare III-37E2-4).

Finally, FrzA gain-of-function and Fzd2 loss of function strongly differs for the expression of three ventral markers. Vent expression decreases in Fzd2 loss of function conditions (Fig. III-37F3, F4) but not when FrzA is overexpressed (Fig. III-37F2). This is particularly obvious for ved, whose expression decreased after Fzd2 loss of function while it strongly increases in almost all cells of the embryo in FrzA gain of function embryos (Fig. III-37G2-G4). In addition, Vox expression is not affected by Fzd2 lost of function (Fig. III-37 H3, H4) but decreased when FrzA is overexpressed (Fig. III-37 H2). Altogether, the analysis of the expression of early molecular markers rules out the hypothesis that FrzA is a simple inhibitor of Fzd2 function. However, the analysis of the effect on Dharma-EGFP showed that the inhibition by FrzA of the β-catenin depends on the presence of Fzd2. Therefore,

FrzA may bind to Fzd2 alone or in combination with Wnt co-receptors and negatively influence their function resulting in the stabilisation of the β-catenin degradation complex.

In conclusion, based on these experiments, I show that at early developmental stages, FrzA does not act, as it has been shown for FrzB, through a direct binding to the Wnt5/8a/11 but may rather function by binding maternal Fzd2 and negatively regulating the maternąl β-catenin.

D. Discussion

1. The link between FrzA *in vivo* function and its expression pattern.

The results from the local injection of FrzA RNA suggest that FrzA works as a short range signal. This is in agreement with other reports on either the human or the mouse SFRP1 genes showing that SFRP1s are located either on the membrane or on the extracellular matrix in cell cultures and that can be released upon addition of heparin (Bafico et al., 1999; Finch et al., 1997). In addition the degree of release from the extracellular matrix depends on heparin concentration (Uren et al., 2000). The short range of FrzA signalling and the evidences that it can locally inhibit the maternal β-catenin pathway suggest its *in vivo* function may be to prevent Wnts of stimulating the animal pole territory at the time of forebrain formation.

2. Possible mechanisms of FrzA on the maternal β-catenin signal inhibition

Maternal β-catenin accumulates specifically in nuclei of dorsal marginal blastomeres as early as the 128-cell stage (Schneider et al., 1996). The region of nuclear accumulation of β-catenin will form the future dorsal axis (Schier and Talbot, 2005). There are not many reports about extracellular cues which can control maternal β-catenin stabilization. In *Xenopus*, Wnt11 activity contributes to early accumulation of β-catenin (Tao et al., 2005). However, zebrafish Wnt11 mutants (*silberblick*) display only defects on morphogenetic movements but not on patterning (Heisenberg et al., 2000). There is another Wnt11 gene in the zebrafish genome (Gates et al., 1999) and future experiments need to clarify if this Wnt11 gene acts as a factor controlling maternal β-catenin.

The data showing that either up-or down-regulation FrzA activity can affect the maternal β-catenin signalling, derive from the analysis of the expression gene known to be downstream target of the β-catenin but not from the analysis of the β-catenin itself. Therefore, I still need to establish that the maternal β-catenin is directly affected either by detecting the un-phosphorylated β-catenin on western blot or by immunohistochemistry to detect the nuclear β-catenin accumulation indicative of the activation of Wnt signalling pathway [Nojima, 2004 #77].

Previous reports on FrzA have suggested several possible mechanims of action for this factor. First, it has been proposed that FrzA may bind to Wnts and prevent them from binding to Fzd2. In zebrafish early embryogenesis it is unlikely that FrzA acts by this mechanism because Wnts either are not expressed at gastrulation stage (for example, Wnt1) or are expressed only at the margin (Wnt3, 5b, 8a, 11), a territory that does not express FrzA. In addition, FrzA is still able to function in the absence of Wnt8 and Wnt11 suggesting that its molecular mechanism of action does not go through a direct binding of Wnts.

The second possible mechanism by which FrzA can down-regulate β-catenin is by binding to Fzd2 and activating the Fzd2 signalling through the Ca2+ pathway. Fzd2 is one of the G-protein coupled receptors that can induce the phospholipase/Ca^{2+} signalling through G-protein molecules (Slusarski et al., 1997). Although a direct interaction between Fzd2 and G-protein molecules has not been proved yet, there is plenty of indirect evidences suggesting the existence of this mechanism. For example, Fzd-mediated signalling can be inhibited by the pertussis toxin, a G protein specific inhibitor (Ma and Wang, 2006; Slusarski et al., 1997). In addition, Fzd2 induced Ca^{2+} signalling has been shown to be able to antagonise the β-catenin signalling (Westfall et al.,

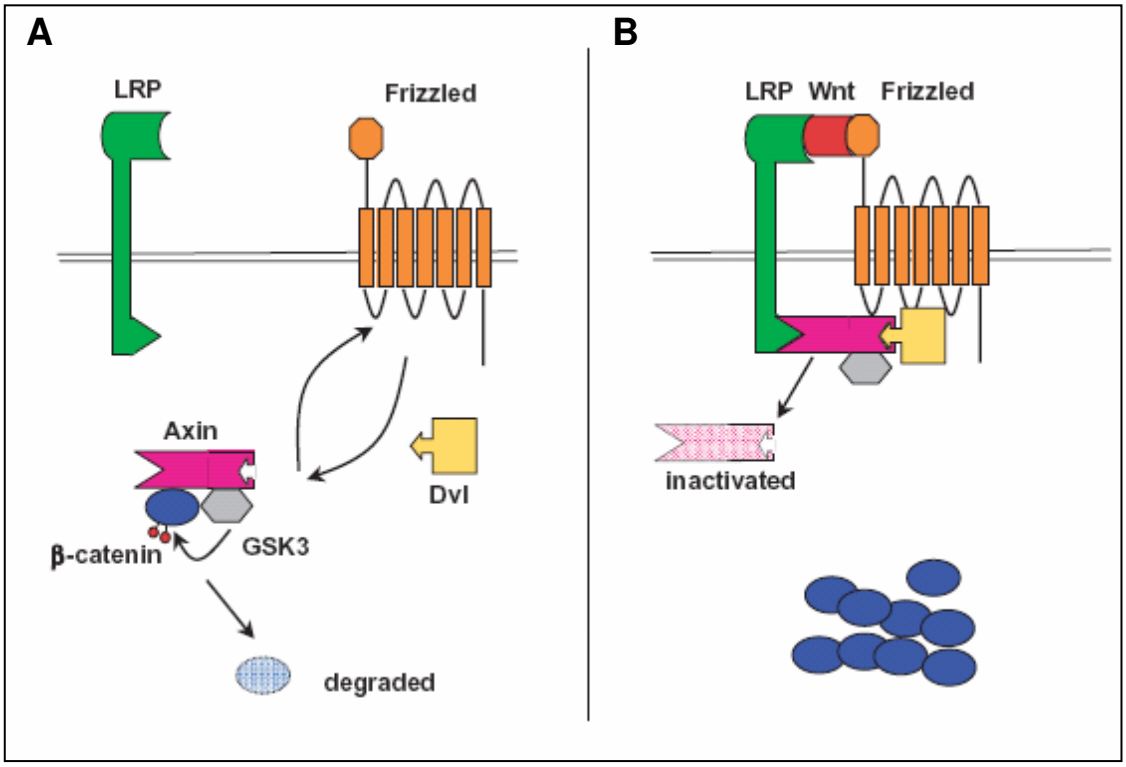


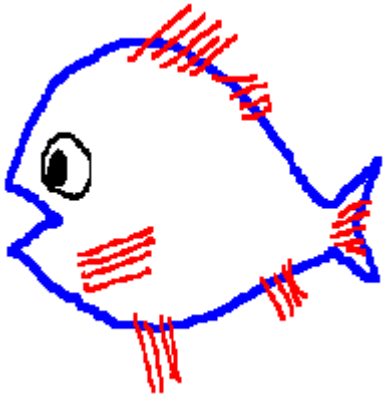
Figure III-38:A model for transducing the Wnt signal by Frizzled and LRP (Photograph from Cong et. al, 2004). (A) Without Wnt signaling, β-catenin is bonded with degradation complex includes Axin and GSK3 and can be phosphorylated by GSK3 then degrade. At the same time, Dishevelled is distributed in the cytoplasm and may have low binding to Frizzled and Axin. (B) When Wnt molecules appear, Wnt can interact with extracellular domains of both LRP and Frizzled receptors and form hetero-oligomers. This extracellular bringing function of Wnt between Frizzled and LRP can recruit Axin (binds to the LRP C-terminal domain) and Dishevelled (binds to the Frizzled intracellular domain) to the membrane and promote the intracellular complex formation among them then subsequently inactivates Axin which may through dephosphorylation.

2003). Finally, a previous report on FrzA showing its function in promoting chicken retinal ganglion cells (RGC) growth cone (Rodriguez et al., 2005) suggested that it was functioning through the activation of Fzd2 signalling. In order to see whether the effect of FrzA on the maternal β-catenin occurs through G protein signalling, I need to analyse the function of FrzA in conditions where G protein activity would be blocked by the use of G protein inhibitor.

The third possible mechanism of action of FrzA may be through the binding to Fzd and the formation of a non-functional complex (Bafico et al., 1999). Fzd has a cytosolic domain which can bind and recruits Dishevelled (Dsh/Dvl) to the membrane region and phosphorylates it. This membrane recruitment of Dsh is a critical mechanism that recruits axin to the membrane and brakes β-catenin degradation complex (Fig. III-38). As a result, if FrzA binds Fzd and form a non-functional complex, Dsh would not be recruited to the membrane and in consequence the maternal β-catenin will be degraded. This mechanism would fit with the result showing a reduction of chordin and Dharma-EGFP expression after FrzA overexpression. It may also explain why Fzd2 knock down phenotype is epistatic on the phenotype of FrzA overexpression for the expression of chordin. However, I do not have a molecular evidence of the formation of this non-functional complex. Nevertheless, a dominant negative form of Fzd2 doesn't seem to downregulate the β-catenin signalling based on the observation of its activity on chordin expression. Since FrzA inhibit the maternal β-catenin signalling mediated by Fzd2 it seems that this would not be through the recruitement of Dsh at the membrane by the Fzd2 C-terminal function. In the meantime, many results show that Fzds interacts with different co-receptors to transfer the Wnt signalling (Cadigan and Liu, 2006). Therefore, FrzA might also bind to Fzd2 and to Wnts co-receptors (for example: LRP6,

Fig. III-38) and inhibit the co-receptors ability to activate the β-catenin signalling. The Fzd co-receptors include LRP6 (low-density-lipoprotein-related protein, (Mao et al., 2001a)) and RYK (atypical tyrosine kinase, (Lu et al., 2004)). Both of them can activate β-catenin through different mechanisms: LRP6 can bind to axin (Cong et al., 2004; Mao et al., 2001b; Tamai et al., 2004) and RYK can bind to dishevelled (Lu et al., 2004), resulting in the disruption of the β-catenin degradation complex. In future experiments, analyzing the effect of the inhibition of these two co-receptors on the activity of FrzA will be required to test this last hypothesis.

Part IV
Discussion



A. Link between cell fate specification and cell movements.

Studies of the non-canonical Wnt pathway show that many molecules involved in this pathway play roles on cell movements without changing cell fate (Solnica-Krezel, 2006). This raises the questions of the relationship between cell fates and cell movement to see whether they are independent or tightly connected to each other. This is difficult to distinguish due to the fact that both cell movements and gene expressions dynamically change at blastula and gastrula stage. During gastrulation, cells not only use a variety of motile cell behaviours to acquire proper positions and sculpt the body plan, but also make specific fate decisions. There are two models that proposed a sequence of cell fates determination and movements (Myers et al., 2002b). The first is the “linear pathway” model for which cell movement behaviours are downstream consequences of cell-fate specification. At the beginning, a cell acquires its positional information then it interprets this information and specifies its fate by expressing cell-type-specific genes. These genes then in turn activate downstream targets defining cell movements. The second model is the “Parallel pathway” model which postulates that cell fates and cell movement behaviours are coordinated by parallel pathways. The difference of this model compared to the first one is that when a cell acquires its positional information, it not only activates a genetic program for cell fates by also activates a separate genetic program that regulates cell movements in parallel.

However, in the real situation both models may be true.

In the experiments where BMP was locally and cell autonomously inhibited by injection of a RNA coding for a truncated-form of the BMP , I showed that the migration abilities of ventrally injected cells was impaired due to a change of cell fates (Fig. II-6I). This result may fit with the linear pathway

model for which change of cell fate occurs first and results in modifications of migration ability as a secondary consequence.

On the other way, transplantation experiments in Rok2 (Rho kinase 2) loss of function conditions (a gene which controls only cell migration but not cell fates) showed that when wild type cells are transplanted into Rok2 mutants embryos or when Rok2 mutant cells are transplanted into wild type embryos, the transplanted cells maintain cell polarity behaviours of their original condition (Marlow et al., 2002). Since lost of Rok2 will not change the cell fates, this result suggests that the impairment on migration ability after Rok2 inhibition is due to a loss of regulation on the cell movement only and is independent of cell fate determination. This result fits the parallel pathway model.

Finally, in many situations it is difficult to separate cell fate and cell movement and the effect on one of this function may affect the other through various cross regulations or even some negative feedbacks.

B. Possible link between Angiomotin like 2, Wnt signalling and the cell migration.

Amotl2 , the Wnt signalling and the cell migration may be linked at the level of the regulation of the intracellular β-catenin. The intracellular free β-catenin cannot only be regulated by the extracellular Wnts or Wnt antagonists' signals but also be regulated by binding to cadherin cytosolic domain (Fig. IV-1). The binding affinity between β-catenin and cadherin can be modulated by tyrosine kinase and phosphatase. Activation of tyrosine kinases includes Src (Roura et al., 1999) that can phosphorylate β-catenin and disrupting the binding of β-catenin to cadherin that results in a loss of cadherin mediated cell-cell adhesion and in an increase in the level of cytoplasmic β-catenin, either by

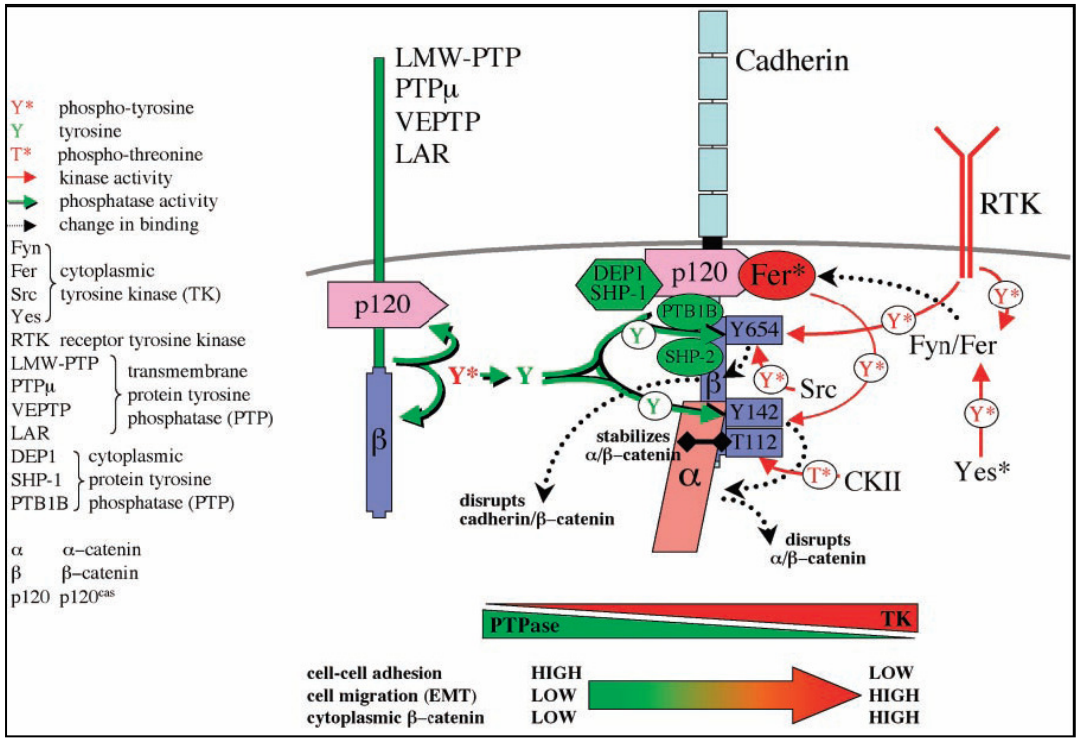


Figure IV-1: Structural and functional regulation of the cadherin-catenin complex by the balance of tyrosine kinase and phosphatase activities (Photograph from Nelson and Nusse, 2004) . Cadherin binds p120 and β-catenin, which in turn binds α-catenin. The integrity of this complex is negatively regulated by phosphorylation of β-catenin by receptor tyrosine kinases (RTKs) and cytoplasmic tyrosine kinases (Fer, Fyn, Yes, and Src), which phosphorylate (red arrows) specific tyrosine residues in β-catenin (Y654, Y142), which leads to dissociation of the cadherin-catenin complex. Integrity of the cadherin-catenin complex is positively regulated by β-catenin phosphorylation by casein kinase II, and dephosphorylation by protein tyrosine phosphatases that bind p120 and β-catenin (green arrows). Changes in the phosphorylation state of β-catenin (bottom) affect cell-cell adhesion, cell migration, and the level of signaling β-catenin.

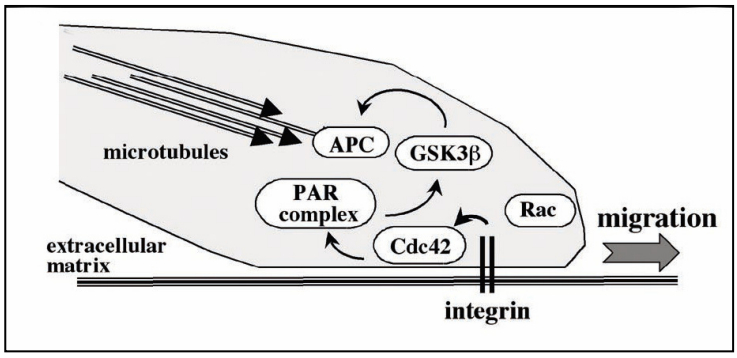


Figure IV-2: Additional roles of Wnt signaling components, adhesion proteins, and APC (Photograph from Nelson and Nusse, 2004) . In addition to a role in targeting β-catenin for degradation, APC also interacts with the plus-end of microtubules at the plasma membrane of migrating cells. Recent studies indicate that APC and microtubules orient the direction of cell migration through a signaling cascade from integrins that bind extracellular matrix; the small GTPase Cdc42; the PAR complex, which contains the scaffolding proteins Par3/Par 6 and an atypical protein kinase C; and serine/threonine kinase GSK-3.

direct release of β-catenin into the cytoplasm or by activating cadherin endocytosis (Le et al., 1999). In contrast, activation of phosphatase stabilizes the cadherin-catenin complex and results in increased cadherin-mediated cell-cell adhesion (Balsamo et al., 1998; Hellberg et al., 2002; Nawroth et al., 2002). As a result, tyrosine kinase activities will regulate the ratio between cadherin-bound β-catenin on the membrane and free β-catenin in the cytoplasm. As the loss of amotl2 will impair the translocation of activated c-Src to the membrane, this suggests that this may affect the level of the cytoplasmic β-catenin.

In addition to the role of src on the β-catenin regulation, Cdc42 GTPase also may be a mediator between Wnt and angiomotin signalling. There are reports showing that Cdc42 GTPase can regulate GSK-3 and adenomatous polyposis coli (APC) to control cell polarity (Etienne-Manneville and Hall, 2001; Etienne-Manneville and Hall, 2003a; Shi et al., 2003). As angiomotin may indirectly activate the Cdc42GTPase activity (Wells et al., 2006) and as Cdc42 is involved in the extracellular signalling by integrin and regulates the activity of Gsk3 and APC, then angiomotin may regulate APC and microtubules and therefore control the direction of cell migration (Fig. IV-2).

C. Link of Wnts and angiomotin with the Cancer

Alteration in cell fate, adhesion, and migration are characteristics of cancer in which cells ignore normal regulatory cues from their environment (Nelson and Nusse, 2004). Loss of control of Wnts signalling (Polakis, 2000) and/or cell-cell adhesion (Christofori, 2003) are involved in cancer induction and progression. Inside the Wnt signalling, up-regulation of β-catenin is found closely related to colorectal cancer (Polakis, 2000). In addition, loss of expression of SFRPs including sFRP1 (FrzA) due to promoter

hypermethylation (Suzuki et al., 2004) is highly frequent in colorectal cancer and may result in an up-regulation of Wnt signalling. Since the Wnt signalling components GSK3 and APC are involved in cell movement regulation, this suggests that the loss of control of Wnt signalling may also be involved in tumorigenesis process like metasis.

Angiomotin is reported as a membrane associated protein that mediates the signalling form angiogenesis inhibitor, angiostatin, which can inhibit blood vessel endothelial cell migration and tube formation *in vitro* (Bratt et al., 2002; Levchenko et al., 2003; Levchenko et al., 2004). Increasing expression of angiomotin and angiomotin like 1/2 are closely linked to breast cancer tumorigenesis (Jiang et al., 2006). Using angiomotin as the target for vaccination can inhibit endothelial cell migration in vitro and can prevent growth of transplanted tumors (Holmgren et al., 2006). The molecular mechanisms of action for angiomotin start to be more understood. Angiomotin is shown to interact with Rich1 GAP which can regulate Rho GTPase activity like Cdc42 or Rac1 (Aase et al., 2007; Wells et al., 2006). The studies on the role of angiomotin provide many information on actin organization, focal adhesion, Rac1 GAP proper localization and also on its contribution to tight junction integrity (Aase et al., 2007; Jiang et al., 2006; Wells et al., 2006). Finally, these data will explain how angiomotin responsible of cell migration ability and will provide way for gene therapy for cancer by an anti-angiogenic strategy (Persano et al., 2007).

D. The benefit of using the zebrafish to study disease.

Using zebrafish as a tool to studies human disease has several benefits. Comparing to the closest model mouse, using zebrafish spends lesser financial support and working staff. Comparing to the most common used

genetics model like *Drosophila* and *C. elegans*, zebrafish provide human closer related organ structure and can be used as markers for drug screen (Lieschke and Currie, 2007). Zebrafish is also suitable for genetic manipulation like mutagenesis (Amsterdam and Hopkins, 2006) and transgenesis (Stuart et al., 1988). As a result, it provides a bridge between vertebrate and invertebrate and has both advantages. These genetic properties offer the opportunities for new chemicals screening on specific mutant zebrafish.

For example, a screening for compound able to rescue gridlock (grd) mutant (a mutation that perturbs the artery/vein balance, resulting in generation of insufficient numbers of arterial cells) identified GS4898 and GS3999 which are similar to phosphatidylinositol 3-kinase inhibitor and vascular endothelial growth factor inducer, respectively (Hong et al., 2006; Peterson et al., 2004). As a result, not only this may produce valuable compounds for therapy but also provide information about the basic biological process involved in the defects under study (Lieschke and Currie, 2007).

In conclusion, the zebrafish would not only be a main stream model on developmental biology but also would be a main biosensor for disease research (Hendrix et al., 2007).

Appendix I- Material and methods

A. Specific methods for the use in the Zebrafish

1. Biological material

Wild type strain was obtained by crossing AB strain and Tü Strain. Breeding conditions are detailed on http://zfin.org/zf_info/zfbook/zfbk.html. One day before injection, a male and a female are put in a tank but are separated by a grid. The grid is removed the day of injection when light is switched on. Eggs are laid, fertilized and then collected. Embryos are treated with Pronase (Protease Type IV dissolved in water at 10 mg/ml) to remove the chorion then washed in water. Embryos are grown in petri dishes coated with 2% agarose in 0.3X Danieau buffer (Stock: 30X NaCl 50.7 g, KCl 0.78 g, MgSO4 1.47 g, Ca(NO3)2 2.1 g, HEPEs 19.52 g, PH 7.3) with 0.5 % penicillin/streptomycin (Gibco, No. 15140-122).

2. Microinjection of Morpholinos or RNAs

Morpholinos oligos (MOs) are synthesized by Genetools Company. MOs are resuspended in a 1X Danieau solution to obtain a 4mM stock solution.

Sense RNAs are synthesized using linearized plasmid (see next section for details) with the RNA transcription kit (Ambion mMessage mMachine kit). After synthesis, the RNA quality is determined by gel electrophoresis and measurement of the optical density (260/280 ratio). Concentration is then calculated (O. D. 260 value of 1 corresponds to 40 µg of RNA). MOs and RNAs are prepared in injection solution: 0,2% phenol red (ICN Biochemicals, No. 34487-61-1), KCl 0,1M in RNAse free water.

The preparations are centrifuged for 2 min, max speed (13,000 rpm/min, eppendorf 5415D) in order to spin down the particles, and loaded into the

micro capillary filament (GC120F-15, Harvard Apparatus) pulled by micropipette puller (Shutter instrument, Model P-97). Injections are done with air-driven Eppendorf 5426 transjector. For whole embryo injection, the MOs or RNAs were injected into the yolk. For one cell injection on the blastomere at 64 or 128 cells stage, the injection position and following procedure are described in specific experimental procedures in Part II of this thesis.

3. Injected sense RNA and Morpholinos

The morpholino oligos (MOs) sequences used in the present study are:

gene	Antisense (AS) or Sense (S)	Sequence (5'>3')	Additional information
Amotl2MO1	AS	CTGATGATTCCTCTGCCGTTCTCAT	cover the ATG condon
Amotl2cMO1(control MO)	S	TACTCTTGCCGTCTCCTTAGTAGTC	Sequence inverted from Amotl2 MO1
MO-FrzA(AGA1)	AS	GGACAAAGATGCAAGGGACTTCATT	cover the ATG condon
MO-FrzA(AGA2)	AS	TGCAGTCAAAGCAACCCCTGAAAAC	cover 5'-UTR region

The other MOs sequences used in the preset study can be found in following references: MO-Ved (Shimizu et al., 2002), MO-Fzd2 (Sumanas et al., 2001), MO-Vent and MO-Vox (Imai et al., 2001), MO-chordin (Nasevicius and Ekker, 2000), MO-FrzB (Agathon et al., 2003), MO-Dkk1 and MO-Wnt8a (Seiliez et al., 2006).

The information for plasmid constructs oligos:

gene	plasmid	Cloning site	Oligos (5'>3') F: forward, R: reverse
FrzA	pCS2+	BamH1/Xho1	F:TTACTCTTAAGGATCCCAGTTTTCAGGGG R:AGTAAGTGGGAGCCTCGAGAATGTTTACTTGAAGACA
FrzA _MO mut	pCS2+	BamH1/EoR1	F:GTCGGATCC**ATGAAAAGTTTGGCGAGCCTTAGT**CTTTGGAGG ATCATCATTTTGA (Solid letters indicate the modified region) R: GAATTCTTACTTGAAGACATTCTCATAGGC

Fzd2-myc	pCS2+_MTC2	EoR1/Xho1	F: TTCGAATTCATGCAGGCGAGTGGAAGTGTG R: ATCCTCGAGAACAGTGGTTTCTCCTTGTC
DNFzd2 (C-terminal Truncated)	pCS2+_MTC2	EoR1/Xho1	F: TTCGAATTCATGCAGGCGAGTGGAAGTGTG R: AGGCTCGAGTCCCGACCAGATCCAGAATC
FrzA-flag	pCMVTAG4A (Stratagene)	BamH1/EoR1	F: GGCGGATCCATGAAGTCCCTTGCATCTTTG R: ATCGAATTCCTTGAAGACATTCTCATAGG
Wnt11-myc	pCS2+_MTC2	BamH1/EoR1	F: GCAGGATCCATGACAGAATACAGGAACTTTCTTC R: CTTGAATTCTTCTTCGAGACGTATCTCTCG
Wnt8-myc	pCS2+_MTC2	BamH1/EoR1	F: GCAGGATCCATGAACCCTTGCCAGATTTTTGC R: CTTGAATTCTTTCCACGACGCGTGTGTTGTC
Wnt8-GFP	pCS2+_MTGFP	BamH1/Cla1	F: GCAGGATCCATGAACCCTTGCCAGATTTTTGCGTC R: TTTAAATCGATATCCACGACGCGTGTGTTGTCTT
E(epithelial)-cadherin (C-terminal Truncated)	pCS2+	BamH1/Xho1	F: GCAGGATCCATGGCTTGTGTAACAACTGTGGGAT R: AGGCTCGAGGGAGTTGCTTTTCCTCCGCA
SrcY527	pCS2+	Xho1/Xba1	F: GATCTCGAGATGGGCAGCAACAAGAGCAAG R: TGATCTAGATTACTTGTACAGCTCGTCCATG

To synthesize 5' capped RNA, the plasmids include pCS2+, pCS2+MT (http://sitemaker.umich.edu/dlturner.vectors/home) and pCS2+MTGFP (Heim and Tsien, 1996) were linearized with Not1 and synthesize with SP6 RNA polymerase. For the other cDNA constructs used in this thesis can be found in following references: Cdc42 G12V (Djiane et al., 2000), N-terminal deletion TCF, β-catenin, N-terminal deletion β-catenin and Dharma-EGFP transgenic constructs are from Hibi's lab (Ryu et al., 2001), VP16-Vent (Onichtchouk et al., 1996), Kaede (Ando et al., 2002), Wnt8 (Kelly et al., 1995), FrzB (Leyns et al.,

1997; Wang et al., 1997a), Dkk1 (Seiliez et al., 2006), BMP2b (Furthauer et al., 1997), Truncated form BMP receptor (TBR) (Hemmati-Brivanlou and Melton, 1994), active form of Smad1 Ras-insensitive, (Kretzschmar et al., 1999).

4. *In situ* hybridization

The injected or wild type embryos are fixed at different stages with 4% paraformaldehyde (PFA) in PBS for 4 ^{o}C overnight, then dehydrated with increasing methanol concentrations and stored with 100% methanol at -20 ^{o}C. RNA probes are synthesized using either linearized plasmid purified on Micorpure-EZ (Amicon, Millipore) and the Microcon Ultracel YM-50 (Millipore) or PCR products purified on Montage PCR column (Millipore). The procedures for synthesizing RNA probes and hybridizations were as protocol published by our team (Thisse et al., 2004).

5. Cell apoptosis assay

The call apoptosis assay for amotl2 MO local injection cells are assayed by Nile blue staining. Nile blue stock solution: 2 mg Nile blue (N-5632, SIGMA) is dissolved in 50 ml distilled water, stirred for 1 hour and filtered with paper (qualitative paper, Grade1, Whatman). For embryo staining, dilute Nile blue stock 64 times in 0.3X Danieau buffer and stained for 30 minutes on the rotation plate (30/min) then washed with 0.3X Danieau buffer 3 times for 10 minutes.

B. Protein-protein interaction and detection

1. Cell transfection and coimmunoprecipitation

Human embryonic kidney cells (293T line) are grown in DMEM (supplemented with 1g/L glucose, 10% Fetal Calf Serum (lot. number 332),

100 U/ml Penicillin and 100 U/ml Streptomycin) and diluted twice a week (1:9 to 1:12). Different combinations of plasmids are transfected with jetPEI^{TM} cationic polymer transfection reagent (Polyplus transfection) or lipofectamine (invitrogene) into 50-60% confluent cells. 36-48 hours after transfection, the cells are washed with PBS, lysed with lysis buffer (50 mM Tris.HCl, 150 mM NaCl, 0.5 % NP40, PH 7.5 with EDTA-free proteinase inhibitor cocktail from Roche, Cat. No. 04693132 001) on ice, and centrifuge 12,000 rpm for 10min at 4 ^{o}C. The supernatants are then transferred to new tubes.

The input expression levels of the proteins are detected in the cell lysates using a equal volume of 2X loading buffer (125 mM TrisHCl, Ph 6.8, 4% SDS, 20% glycerol, 0.004% bromphenol blue, 5% 2-mercaptoethanol). The samples are boiled for 5 minutes and loaded into an SDS-PAGE acrylamide gel.

The coimmunoprecipitation assay are performed as followed: cell lysates are supplemented with 15 l agarose conjugated anti-myc antibody (c-Myc 9E10: sc-40 AC, D-2607, santa cruz Biotechnology, INC) or agarose conjugated anti-flag M2 antibody (A2220, SIGMA) and mixed end-over-end for 4 ^{o}C overnight. Immunoprecipitates (400 ml) are washed 3X in lysis buffer. They are resuspended in 2X loading buffer, boiled and loaded into SDS-PAGE acrylamide gel.

2. Western blotting

10% polyacryamide gel are used to separate the cell extract in 1X migration buffer (1L of 5X stock solution: 15.1 g Tris base, 94 g glycine, 50 ml 10% SDS) for 1 hour at 50 mA. The transfer is performed using a PVDF membrane (Amersham Hybond-P). The membrane is pre-wet with 100% methanol and incubated in transfer buffer (3.03 g Tris base, 14.4 g Glycine and 200 ml methanol in 1L, PH 8-8.5) for 20 minutes. The transfer is performed in

transfert buffer for 2 hours at 100 V using Mini-PROTEAN Tetra electrophoresis system (Bio-rad). After transfer, the membrane is blocked with 5% de-fat milk in TBST(20 ml 1M TrisCl, 8 g NaCl and 2.5 ml 20% Tween 20, PH7.6) for 1 hour and washed 3X with TBST. The membrane is then incubated with HRP conjugated anti-myc antibody (1: 1,000, c-Myc 9E10: sc-40, F0706, SANTA CRUZ) or HRP conjugated anti-flag M2 antibody (1: 1,000, A8592-1MG, SIGMA) in TBST for 1 hour and washed 3X in TBST. Finally, the membrane is rinsed with 1X PBS. The detection is performed using the ECL system (Amersham ECL Plus Western Blotting Detection Reagents, RPN 2132) and Kodak films (Biomax XAR film, F5388-50EA).

Appendix II- Abbreviation

ADF	Actin-Depolymerizing Factor
Alk8	Activin receptor-like kinase 8
Amotl2	Angiomotin like 2
AP	Anterior-Posterior
APC	Adenomatous Polyposis Coli
Arp	Actin-Related Proteins
BAMBI	BMP and Activin receptor Membrane Bound Inhibitor
β-TrCP	β-transducin repeat-containing proteins
BMP	Bone Morphogenetic Protein
BRE	BMP response element reporter
Cdc42	Cell division cycle 42
cDNA	complementary Deoxyribonucleic acid
CE	Convergence Extension
Chd	Chordin
CRD	Cystine-Rich Domain
CtBP	C-terminal-Binding Protein
cyc	*cyclops*, mutant for Nodal related 2 gene
Cyp11a1	cytochrome P450, subfamily XIA, polypeptide 1
Cyp26a1	cytochrome P450, subfamily XXVIA polypeptide 1
DEL	Deep cell layer
DEP	Disheveled, EGL-10, Pleckstrin
DIX	dishevelled and axin
DKK1	Dickkopf 1
Dlx3	Distal-less homeobox gene 3b
DNA	Deoxyribonucleic acid
dpf	day post fertilization
Dpp	Decapentaplegic
Dsh/Dvl	Dishevelled
DV	Dorsal-Ventral
Ecdh1	Epithelial cadherin 1
ECdh1-TC	Truncated from of Epithelial – cadherin
ECM	Extracellular Matrix
EGF-CFC	Epidermal Growth Factor - (Cripto, FRL-1, and Cryptic)
EMT	Epithelial Mesenchymal Transformation
Ena/VASP	Enabled/Vasodilator-Stimulated Phosphoprotein
ERK	Extracellular signal-Regulated Kinases
Erm	Ets-related protein

ES	Early somitogenesis
Ets	Erythroblast transformation specific
Eve1	even-skipped-like1
EVL	Enveloping Layer
Fgf	Fibroblast growth factor
FGFR	Fibroblast Growth Factor Receptor
flk	floating head
FoxA3	Forkhead box A3
Foxi	Forkhead box I
Fzd2	Frizzled receptor 2
GAP	GTPase-Activating Protein
GDI	Guanine nucleotide Dissociation Inhibitors
GDP	Guanosine Diphosphate
GEF	Guanine nucleotide Exchange Factor
GFP	Green Fluorescent Protein
Grb2	Growth factor receptor-bound protein 2
Gsc	Goosecoid
GSK3	Glycogen Synthase Kinase 3
GTP	Guanosine-5'-triphosphate
HDAC1	histone deacetylase 1
Hgg1	cathepsin Like 1b
HMG	High-Mobility Group
hpf	hours post fertilization
IP	Immunoprecipitation
JNK	Jun N-terminal kinase
kny	*Knypek*, mutant for heparin sulfate proteoglycan gene
LEF	Lymphoid Enhancer binding Factor-1
LRP	low density lipoprotein-Related Protein
LV	Lateral View
MAD	Mothers Against Decapentaplegic
MAP	Mitogen-Activated Protein
MBT	Mid-Blastula Transition
MDCK	Madin-Darby Canine Kidney
MO	Morpholino oligos
MS	Middle Somitogenesis
MTOC	Microtubule-Organizing Center
MyoD	Myogenic differentiation
NCEZ	No Convergence or Extension Zone
ntl	No tail

NTR	Netrin
Oep	One eye pinhead
PAK	P21-Activating Kinases
PAR	Partitioning-defective protein
PCP	Planar Cell Polarity
PDGF	Platelet-Derived Growth Factor
PDZ	Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and Zonula occuldens-1 protein (zo-1)
Pea3	Polyomavirus enhancer activator-3
PI3K	Phosphoinositide 3-Kinase
PIP2	Phosphatidylinositol bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKB	Protein Kinase B
PKC	Protein Kinase C
ppt	*Pipetail,* mutant for Wnt5 gene
PTEN	Phosphatase and Tensin homolog
Rap1	RAS-related proteins 1
RGC	Retinal Ganglion Cell
RNA	Ribonucleic acid
ROR2	Receptor tyrosine kinase-like Orphan Receptor 2
SEF	Similar Expression to Fgf genes
SFRP	Secreted Frizzled Related Protein
sib	*Silberblick,* mutant for Wnt11 gene
Spry	Sprouty
spt	*Spadetail,* mutant for T-box transcription factor
Src	Sarcoma
STAT	Signal Transducers and Activator of Transcription
swr	*swirl*, mutant for BMP 2b gene
TBR	Truncated BMP Receptor
TCF	T-Cell-specific transcription Factor
TGFβ	Transforming Growth Factor beta
Tll1	Tolloid
TNF	Tumor Necrosis Factor
tri	*trilobite*, mutant for vang-like 2 gene
Tsg	Twisted gatstrulation
UV	Ultra Violet
VE	Visceral Endoderm
Ved	Ventrally expressed dharma/bozozok antagonist
Vent	Ventral expressed homeobox

Vox	Ventral homeobox
WASP	Wiskott-Aldrich Syndrome Protein
WAVE	WASP family Verprolin homologous
Wg	Wingless
WIF	Wnt Inhibitory Factor
XFLRT3	Xenopus Fibronectin-Leucine-Rich transmembrane protein 3
YSL	Yolk Syncytial Layer

Appendix III- Bibliography

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Résumé: "Contrôle des mouvements cellulaires et de la mise en place du patron embryonnaire précoce par les facteurs Angiomotin like 2 et FrzA chez l'embryon de poisson zèbre (*Danio rerio*). "

Le contrôle des movements cellulaires et de la détermination cellulaire est critique pour le développement de l'embryon. Dans cette étude j'analyse la fonction de deux gènes, Angiomotine like2 (Amotl2) et FrzA (SFRP1, Secreted Frizzled Related Protein 1) et je démontre qu'ils jouent un rôle essentiel dans le contrôle de la détermination cellulaire et des mouvements cellulaires au cours de l'embryogenèse du poisson zèbre (Danio rerio).

L'étude de la fonction d'Amotl2 indique qu'il est impliqué dans le contrôle des mouvements cellulaires incluant les mouvement d'épibolie et de convergence et extension. Au niveau moléculaire je démontre qu'Amotl2 interagit préférentiellement et facilite la translocation membranaire de la forme phosphorylée de c-Src. Ceci résulte en la régulation de l'architecture de la membrane et dans le contrôle de la formation des lamellipodes. De plus je montre que l'expression d'Amotl2 est sous le contrôle de la voie de signalisation FGF et que ce gène est aussi requis pour la sécrétion de Wnt8.

L'étude parallèle sur FrzA révèle que ce membre de la famillle SFRP est aussi requis pour le contrôle des mouvements cellulaires et de la mise en place du patron embryonnaire. L'analyse de sa fonction indique que ce gène régule négativement l'activité maternelle de la β-caténine. FrzA semble agir de manière paracrine et être un facteur agissant à courte distance. J'ai établi que le mécanisme fonctionnel de FrzA est différent de celui de FrzB et DKK1 et qu'il n'agit pas via une régulation de l'activité zygotique de Wnt8. Enfin, je montre que l'inhibition de la β-caténine maternelle par FrzA est médiée par le récepteur Frizzled 2.

Summary: "Control of cell movements and early patterning of the zebrafish (*Danio rerio*) embryo by Angiomotin like 2 and FrzA".

Control of cell fates and cell movements is critical for the development of the embryo. In this study I analyzed the function of two genes, Angiomotin like 2 (Amotl2) and FrzA (SFRP1, Secreted Frizzled Related Protein 1) and demonstrated that they play an essential role in controlling the cell fates and cell movements during the embryogenesis of the zebrafish (Danio rerio).

The study of Amotl2 function indicates that it is involved in the control of embryonic cell movements, including epiboly and convergence extension movements. At the molecular level I demontrated that Amotl2 interacts preferentially with and facilitates outward translocation of the phosphorylated c-Src, which may in turn regulates the membrane architecture resulting in the control of formation of lamellipodia. In addition I show that the expression of Amotl2 is under the control of the FGF signaling pathway and that this gene is also required for the patterning of the embryo. Preliminary data suggest that Amotl2 is required for the secretion of Wnt8.

The parallel study on FrzA revealed that this member of the SFRP family is also required for proper cell movements and embryo patterning. Analysis of FrzA function indicates that this gene negatively regulates maternal β-catenin signaling. FrzA appeard to act in a paracrine manner and acts at short range. I established that the functional mechanism of FrzA is different from FrzB and DKK1 and that it does not act by regulating the zygotic activity of Wnt8. Furthermore, I showed that the inhibition of the maternal β-catenin signaling by FrzA is mediated by the Frizzled 2 receptor.