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Sphingosine 1-phosphate signaling in zebrafish heart and endoderm development

by

Nicholas Francis Osborne

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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For Mom and Dad... the most important teachers I've ever had.

Acknowledgements

I would like to thank all the people who have been members of the Stainier lab during my stay. Thank you for all the discussions, constructive criticism and vital assistance with my lab work. In particular, I would like to thank my advisor Didier Stainier for his excellent guidance both in science and in thinking about my future plans.

I also thank my committee members: Jason Cyster for lending his expertise with sphingosine 1-phosphate signaling and for providing a non-developmental outlook to my project, Shaun Coughlin for providing his expertise in G protein-mediated signaling and his enthusiasm for the project and Louis Reichardt for lending his understanding of developmental biology and helpful advice. I thank Susan Schwab for her assistance with measuring sphingosine 1-phosphate levels in zebrafish embryos and Sean Sweeney and Graeme Davis for sharing unpublished reagents and ideas about *two of hearts,* as well as *spinster-like* genes in general. To Lisa Magargal: thank you for going above and beyond the call of duty in helping me get my dissertation turned in from a distance.

Many thanks go out to family for all their love and support throughout the many years I have been working towards this goal. To my sisters, Erica and Heather, thank you for taking time to call and make sure I was always in good spirits. To my mother, Stephanie, and my father, Richard... the dedication says it all.

Finally I would like to acknowledge my partner, Courtney Babbitt. Courtney has contributed her own scientific expertise and advice to my work. More importantly, however, Courtney has given me the support and love I needed to finish this long process. She has made me a better thinker and a more confident scientist.

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Sphingosine 1-phosphate signaling in zebrafish heart and endoderm development

Nicholas Francis Osborne

ABSTRACT

One of the first morphogenetic processes in vertebrate heart development is the assembly of the midline primitive heart tube from two fields of precardiac mesodermal cells. How these two groups of anterior lateral mesodermal cells migrate to the embryonic midline to form a single heart tube is a question of great interest. One developmental system that has contributed to our understanding of heart tube assembly is the zebrafish. Numerous zebrafish mutants have been isolated that cause defects in the migration of the precardiac mesodermal cells to the midline, therefore disrupting formation of the primitive heart tube. One such mutant, *miles apart (mil)*, was shown to encode an orthologue of the mammalian G protein-coupled receptor, $S1P_2$. $S1P_2$ is a member of a family of receptors that specifically recognize sphingosine 1-phosphate (S1P) as their ligand. In the first chapter of this dissertation I review the current understanding of how S1P is regulated and then how it mediates signaling by its receptors. I discuss the biology of this receptor family with respect to downstream signaling partners as well as some of the biological processes affected by the receptors.

In the second chapter, I present data showing that the precardiac mesoderm migration defect seen in *mil* mutant zebrafish embryos is due to a defect in the morphogenesis of the anterior endoderm, a tissue known to be required for migration of

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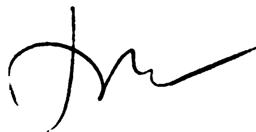
the precardiac mesoderm in zebrafish. Furthermore, I show that these endodermal defects cause craniofacial development defects in *mil* mutants.

In the third chapter, I introduce another zebrafish mutant *two of hearts (toh)* and show that *toh* is a necessary component in signaling via Mil, but not by another related S1P receptor. Furthermore, the *toh* locus encodes a putative twelve pass transmembrane transporter related to the *Drosophila spinster* gene. These data represent the first connection between a vertebrate *spinster-like* gene and a specific signaling pathway. These findings also demonstrate that *toh* is a novel component in signaling via an S1P receptor.



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CHAPTER 1: Sphingosine 1-phosphate signaling

1.I. Introduction

Over the past several decades the understanding of the role lipids play in biological processes has increased a great deal. One group of bioactive lipids in particular has seen a recent explosion in data regarding their biological function: lysophopholipids. Lysophospholipids, and in particular sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) (Figure 1.1A), are now known to affect a wide variety of both developmental and physiological processes. S1P, for example, has emerged as a new target for drug therapy and has been widely investigated using both in vitro and in vivo techniques. The manner in which this lipid functions both during development and later in life has become a critical question in a variety of fields, including immunology, neurobiology and cardiovascular biology.

In order to understand the place S1P occupies in the regulation of biological processes one must first understand certain factors regarding the S1P molecule. These factors include (1) how S1P is synthesized and degraded, (2) how the synthesis of S1P is regulated, (3) how S1P is transported through the body, (3) how S1P interacts with cellular signaling machinery, (4) how signaling downstream of S1P affects cellular responses and (4) the implications of these cellular responses to living organisms. In this chapter I investigate these questions to illuminate the current state of understanding of S1P and its biological roles in vertebrate organisms.

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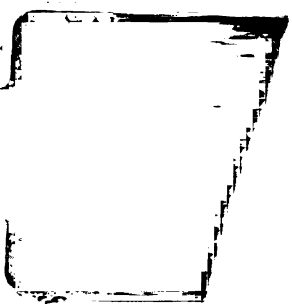
1.II. Sphingosine 1-phosphate biosynthesis



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The first step in S1P mediated signaling is the production of S1P itself. Therefore, in order to understand the initiation of S1P signaling, it is important to understand how this lipid is synthesized. Once present, however, there must also be mechanisms in place to limit or attenuate the ability of S1P to activate signaling. Thus, the mechanisms by which S1P is degraded are also important to the in vivo functions of this lipid. In this section, I discuss what is known about the biosynthetic pathways mediating both synthesis and degradation of S1P.

1.IIa. Sphingosine 1-phosphate synthesis

The initial steps of S1P synthesis in vertebrates are thought to be primarily mediated by the degradation of the membrane component sphingomyelin (SM) (Reviewed in Saba and Hla, 2004; Figure 1.1B). SM is metabolized into ceramide via the activities of a family of sphingomyelinases. Ceramide, in turn, is deacetylated to the lysolipid sphingosine by ceramidase activity. Finally, sphingosine must be phosphorylated in order to generate S1P. This phosphorylation step is carried out in mammals by two isoforms of the enzyme sphingosine kinase. In addition to degradation of membrane lipids, S1P can also be synthesized de novo from small metabolites via ceramide biosynthesis (Reviewed in Pyne and Pyne, 2000). However, the impact of de novo S1P synthesis on signaling in vertebrates is unclear.

1.IIb. Degradation of sphingosine 1-phosphate

The second branch of S1P biosynthesis is the pathways by which S1P is broken down to attenuate the ability of this lipid to signal. There are two degradative pathways that lead

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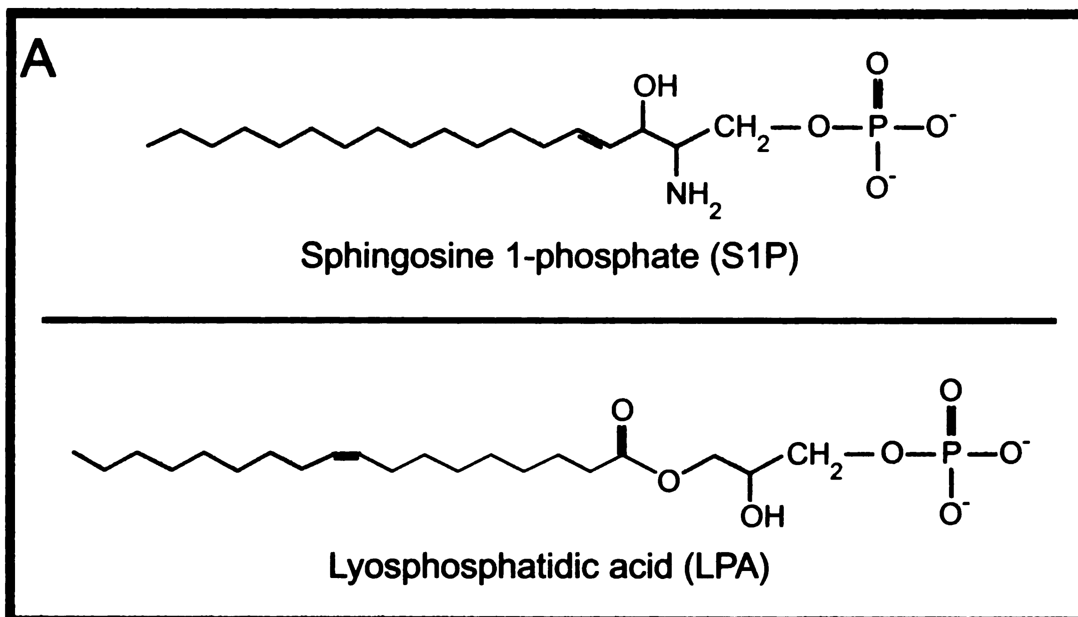
Figure 1.1



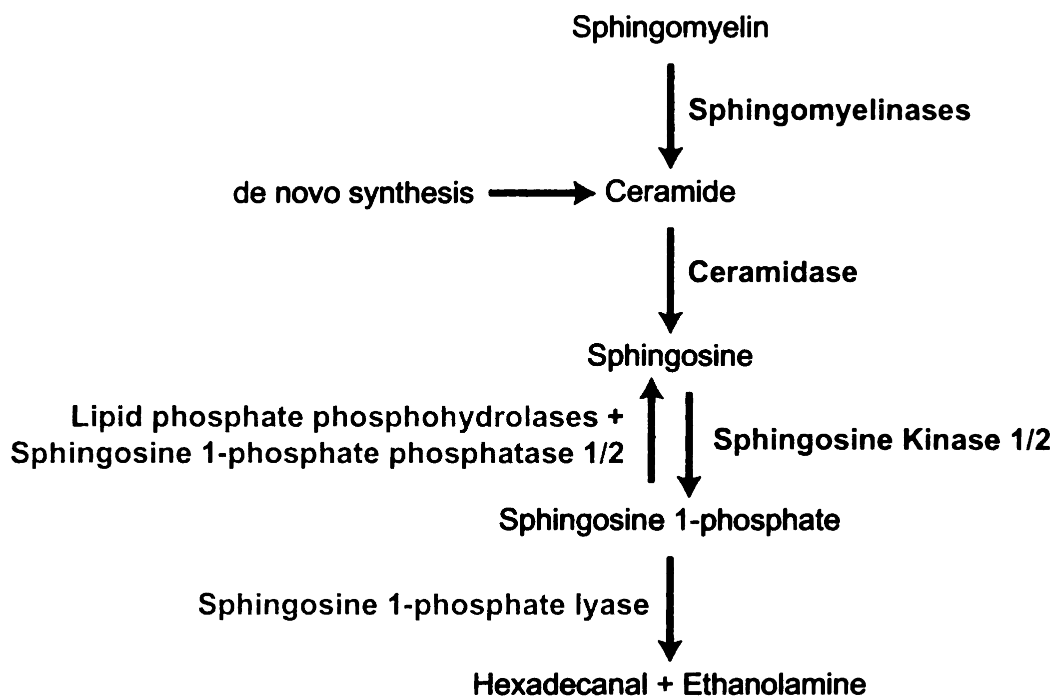


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Figure 1.

(A) Che

enzyme

Figure 1.1: Lysophopholipids and sphingosine 1-phosphate biosynthesis

(A) Chemical structure of S1P and LPA. (B) S1P biosynthetic pathway. (Green) enzymes involved in synthesis of S1P. (Red) enzymes involved in S1P degredation.

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to attenuation of S1P signaling (Reviewed in Saba and Hla, 2004; Figure 1.1B). First, S1P can be dephosphorylated by the activity of phosphatases. In both vertebrates and lower eukaryotes there are non-specific and specific sets of phosphatases that mediate the conversion of S1P to sphingosine. A family of non-specific lipid phosphate phosphohydrolases (LPPs) can dephosphorylate S1P. However, these LPPs dephosphorylate a variety of other phospholipids, including LPA, phosphatidic acid and ceramide 1-phosphate and so constitute a fairly non-specific method of degrading S1P.

As mentioned above, however, there are also phosphatases that are specific to S1P. S1P phosphatase was first discovered in yeast and, subsequently, vertebrate S1P phosphatases were identified on the basis of protein sequence homology. In humans, there are two isoforms of S1P phosphatase, *hSPP1* and *hSPP2*. These isoforms are encoded by different loci and do not have entirely overlapping expression pattern, suggesting non-redundant functions. Combined, the LPP and SPP families of phosphatases constitute the first S1P degradative pathway, namely the reversible dephosphorylation of S1P to sphingosine.

However, another pathway regulates a less reversible degradation of S1P. This second pathway of S1P degradation involves the cleavage of S1P to yield hexadecanal and phosphoethanolamine. A specific S1P lyase regulates this cleavage. The irreversible degradation of S1P to hexadecanal and phosphoethanolamine may also be a critical mechanism for cells to generate the building blocks for lipid membrane components as well as to attenuate the effects of S1P signaling.

1.III. Sphingosine Kinase 1 and 2

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The rate limiting step in the synthesis of S1P is the phosphorylation of sphingosine to S1P. As was stated above two, SK isoforms catalyze this phosphorylation step. These kinases share strong sequence homology in their kinase domains but outside of that domain are quite divergent in sequence. A great deal of work has been done studying the activities of these two kinases because they are the point in S1P biosynthesis that seems most sensitive to outside signaling pathways. As a result much of the regulation of S1P production seems to be controlled at the level of the two SKs.

Therefore, understanding the properties of the SK isoforms helps in the understanding of when, where and how S1P is produced. In this section, I discuss the different characteristics that allow the SK isoforms to be differentiated from one another and the effects of extrinsic signaling on the activity of these kinases.

1.IIIa. Differentiating the sphingosine kinase isoforms

SK1 and SK2 have unique characteristics allowing them to be differentiated. The activities of the two SK isoforms can be easily distinguished based on the conditions under which they best function (reviewed in Olivera and Spiegel, 2001). SK1 activity is highest in low salt concentrations in the presence of Triton X-100, a detergent that inhibits the activities of SK2. Furthermore, SK1 function is associated with both cytosolic and membrane fractions of cells. Membrane localization after activation of SK1 has been shown to have positive effects on the activity levels of this enzyme in some cases (Johnson et al., 2002). SK2, on the other hand, functions best in the absence of detergent and at high salt concentrations. Under these conditions SK1 is non-functional. Activity of SK2 can be found solely in the cytoplasmic fractions of cells and no change in

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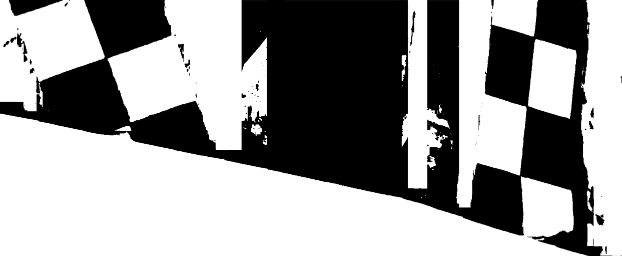

localization has been detected upon activation of this enzyme. The reason for the differing conditions favored by SK1 and SK2, and the relevance of these differences to SK function within cells and animals is unclear. However, these differences have allowed the function of SK1 and SK2 to be separated biochemically and have, therefore, allowed some dissection of other differences between these enzymes.

The second important difference between SK isoforms is that they have dissimilar patterns in substrate recognition. The SK2 isoform appears to recognize many more substrates for phosphorylation than SK1, including the important pharmacological agent FTY720 (Kharel et al., 2005). The biological and clinical importance of FTY720 will be discussed in greater detail later. SK1, in contrast to SK2, is quite specific to sphingosine. This specificity even precludes the recognition of close relatives of sphingosine that are recognized by SK2 as a substrates for phosphorylation.

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1.IIIb. External control of Sphingosine Kinase activity

Early biochemical studies of S1P production demonstrated that numerous extrinsic factors affect the level of S1P production through the SKs. One major group of signals that lead to high SK activity is growth factors. For example TGFβ, PDGF, EGF and VEGF signaling all result in increased S1P production in certain cells via activating SK (Hait et al., 2005; Olivera and Spiegel, 1993; Shu et al., 2002; Yamanaka et al., 2004). In addition to growth factors, there are several immunogenic signals that seem to upregulate SK function. One such immunogenic signaling molecule that activates SK production of S1P is TNFα (Xia et al., 1999). Finally, activation of SK by FcεRI crosslinking has been shown to be critical in the activation of mast cells (Jolly et al., 2004). The activation of

SK is necessary for mast cell degranulation and chemotaxis downstream of FcεRI crosslinking. While there are many other molecules that affect S1P production via activation of SK, these examples give a clue as to the variety of signaling pathways that activate S1P production.

1.IV. Transport of sphingosine 1-phosphate

Once S1P has been generated via SK activity, the next step in signaling is to ensure that S1P reaches its site of action. It is known that S1P can act at a distance within living organisms. However, because S1P is a fairly hydrophobic molecule that must cross a hydrophilic environment, special mechanisms must be employed to ensure S1P can travel those long distances. In adult vertebrates, for instance, S1P is found at high concentrations in the blood serum (Igarashi and Yatomi, 1998) and must be able to travel through this aqueous environments without falling out of solution. In order to remain in this aqueous environment, S1P must be held in solution by binding to carrier proteins. In serum, S1P is found bound to both albumin and high density lipoprotein complexes (Sachinidis et al., 1999; Yatomi et al., 2000). These proteins bind the hydrophobic tail of S1P, allowing the lipid to be carried, unencumbered, through hydrophilic environments.

In the developing embryo, the mechanism by which S1P is made available to the surface of cells is less clear. In many cases during development there is no known carrier protein expressed. How S1P acts in these cases is a mystery. However, recent evidence has shown that one manner in which S1P may be made available to external receptors is through release of SK1 into extracellular spaces (Ancellin et al., 2002). Ancellin et al. have shown that the activity of SK1 can be found on the outer leaflet of the plasma

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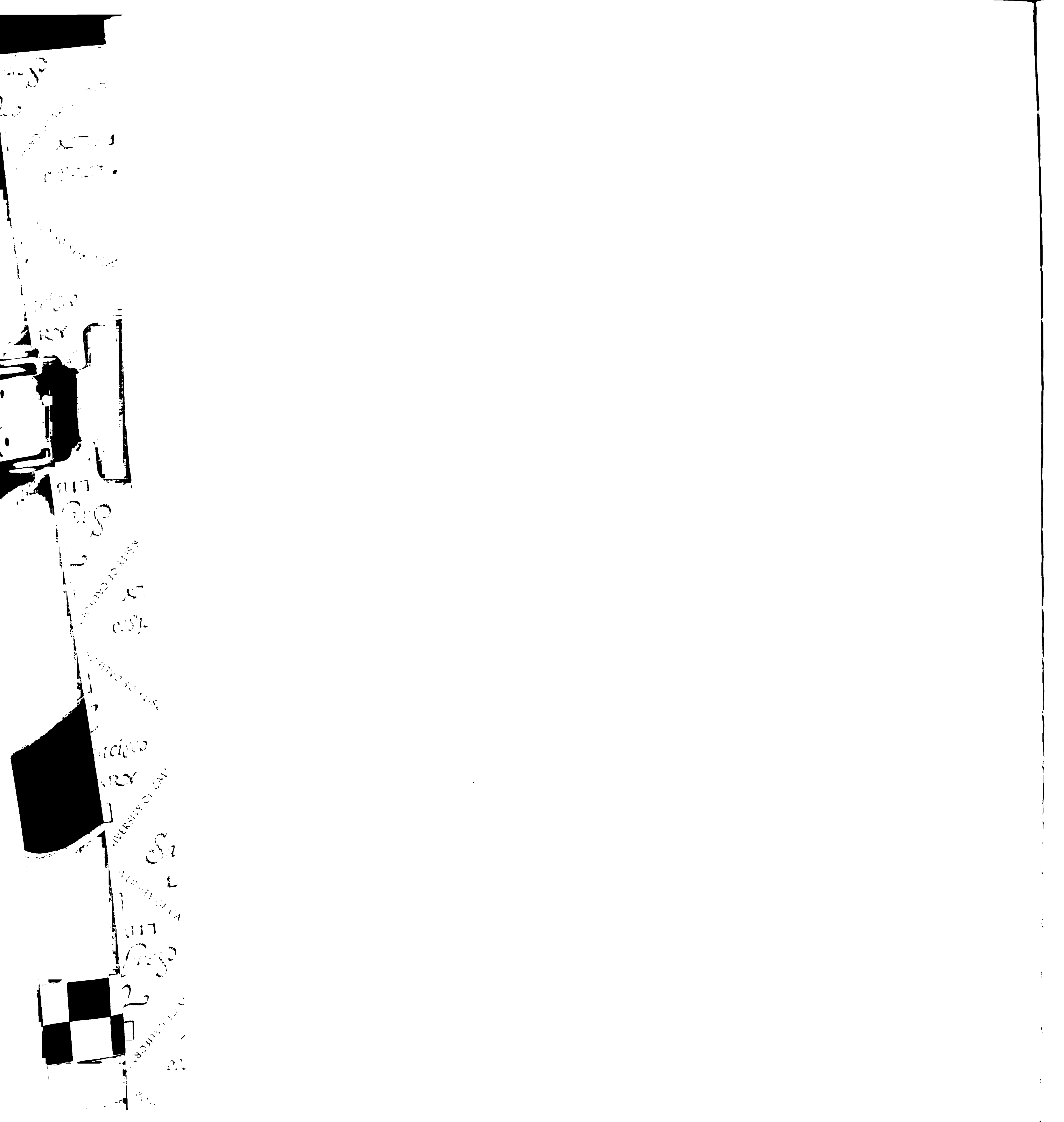

membrane or in the extracellular space of certain cell types. Although this was only e shown in human umbilical vascular endothelial cells (HUVECs), it is possible that many cells have the ability to export their SK1 enzyme activity. The export of SK1, however, is controversial due to the absence of proof that the SK1 protein itself is found in extracellular spaces.

It is also possible, however, that during embryonic development S1P does not act at a distance at all. If S1P cannot act at a distance, it may act in an autocrine or short range paracrine fashion within the membrane of the cells producing S1P and their immediate neighbors. Such a short range system would relieve the necessity for carrier proteins in developing embryos.

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1.V. Sphingosine 1-phosphate receptors

I have discussed the mechanisms allowing S1P to act over long distances or act in an autocrine or paracrine fashion. Implicit in these statements, however, is the idea that S1P acts at the plasma membrane of cells rather than functioning as an internal second messenger molecule. Almost all the cellular effects of S1P, either produced internally or supplied exogenously, can be explained via the activation of S1P specific cell surface receptors. In the next section, I discuss the identification of the S1P receptor family and the intracellular signaling components that interact with S1P receptors.

1.Va. Identification of sphingosine 1-phosphate receptors

The first sphingosine 1-phosphate receptor identified was discovered in a screen examining transcripts that were upregulated in endothelial cells activated with phorbol

12-myristal 13-acetate (PMA) (Hla and Maciag, 1990), an activator of protein kinase C. PMA induced endothelial cells were found to have high levels of a transcript encoding an orphan G protein-coupled receptor (GPCR). The transcript was named *endothelial differentiation gene 1 (Edg1).* Although the gene encoded by the *Edg1* transcript was identified as a GPCR, the ligand remain a mystery until a related receptor gene, *ventricular zone gene-1 (vzg-1)*, later called *Edg2*, was shown to encode a receptor that specifically responded to the lysophospholipid LPA (Hecht et al., 1996). Although the EDG1 receptor did not respond to LPA like VZG-1, Lee et al. were able to show that EDG1 specifically responded to S1P, which is structurally similar to LPA (Lee et al., 1998; Figure 1.1A).

With the identification of EDG1 and EDG2 as lipid receptors several other related receptors were identified as responding to lysophosholipids based on sequence similarity. There are currently identified five S1P and three LPA receptors in the EDG family. In 2002, S1P and LPA receptors were given a unified nomenclature (Chun et al., 2002). All S1P receptors were given the designation $S1P_x$ and LPA receptors the designation LPA_x, where x indicates the order of identification. EDG1 became $S1P_1$ and VZG1/EDG2 became LPA_1 as founding members of their respective subfamilies. From this point on all receptors will be referred to by this accepted nomenclature. The combined family of S1P and LPA receptors is, however, still commonly referred to as the EDG family. Figure 1.2 gives a phylogenetic comparison of many of the known EDG family receptors. As would be predicted the S1P and LPA receptor subfamilies each form a monophyletic group, suggesting an early divergence of function between the two subfamilies.

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Figure 1.2

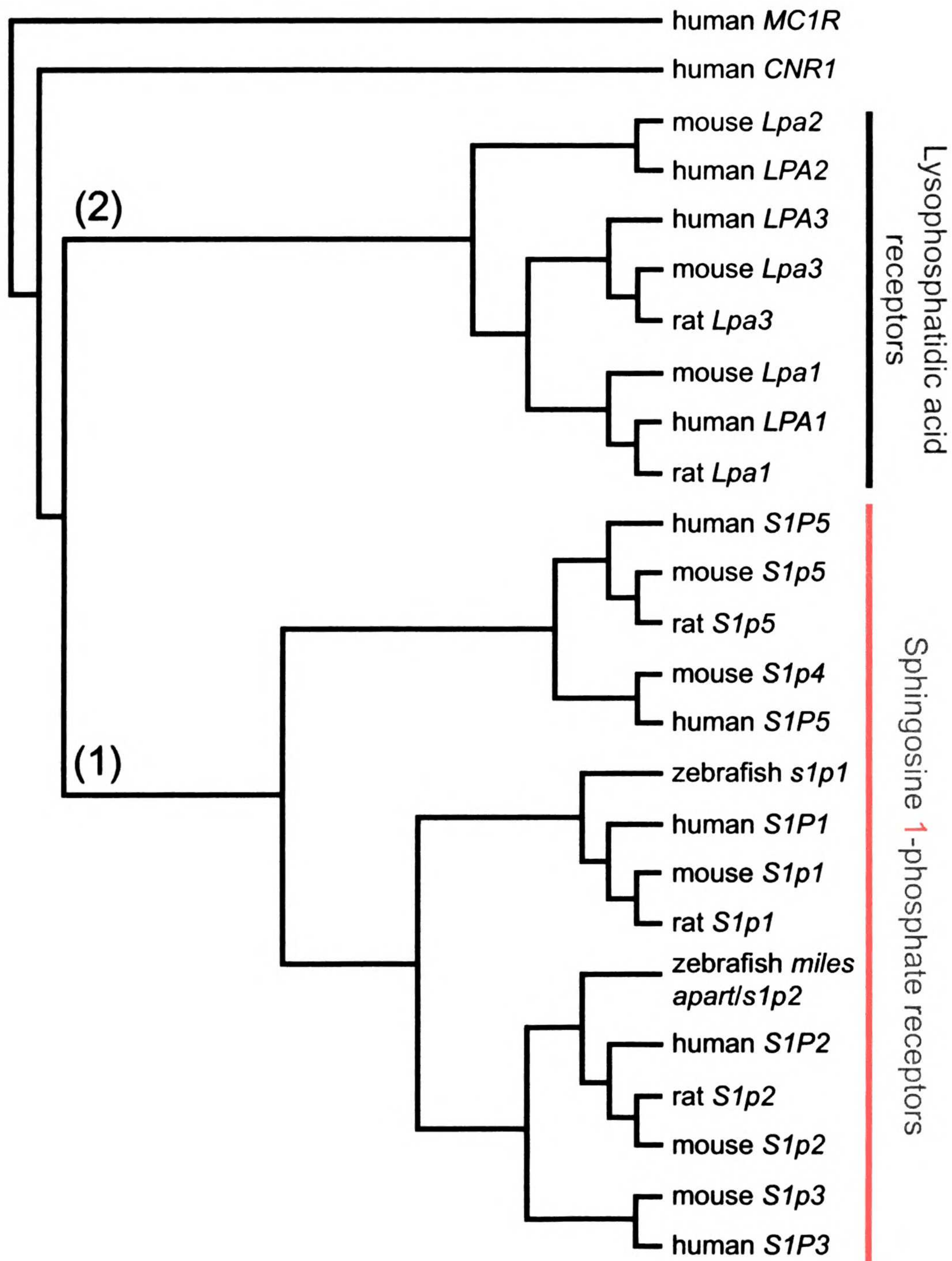

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Figure 1.2: Phylogenetic comparison of EDG genes

DNA coding regions were acquired from GenBank and aligned in ClustalX with gap opening and extension penalties set to default. Phylogenetic reconstruction was carried out in PAUP* 4.0b10 using parsimony as the optimality criterion. Because of the conserved nature of gap regions between closely aligning sequences, gaps were considered to be phylogenetically informative and were counted as a fifth character state. *MC1R* encodes the human melanocortin 1 receptor and *CNR1* encodes the cannabanoid receptor 1, distantly related G protein-coupled receptors used as outgroups. Branch points for the sphingosine 1-phosphate receptor (1; red) and lysophosphatidic acid receptor (2; green) families are indicated.

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Of the five S1P receptor isoforms only three of the receptors show widespread expression. While $S1P_4$ and $S1P_5$ have limited expression, in hematopoetic and lymphoid tissues and in the spleen and adult brain, respectively (Van Brocklyn et al., 2000; Yamazaki et al., 2000), $S1P_1$, $S1P_2$ and $S1P_3$ are widely expressed during embryogenesis and in adult animals. Because the majority of research on S1P receptors has dealt with these three receptor isoforms, I will focus the rest of my discussion on $S1P_1$, $S1P_2$ and $S1P_3$.

As stated above, all of the known S1P receptors belong to the GPCR super-family of cell surface receptors. Although the three major S1P receptors all belong to the same family, they have quite different signaling properties. In the next section I explain how three seemingly similar receptors couple to unique downstream signaling to produce a variety of cellular responses.

1.Vb. Signaling downstream of S1P receptors

One of the interesting characteristics of S1P as a signaling molecule is the wide variety of responses it elicits in different cells types. This versatility in signaling is the result of the fact that each of its receptors has very different downstream signaling potential. In this section, I investigate some of the specific ways in which diverse signaling responses are activated downstream of $S1P_1$, $S1P_2$ and $S1P_3$.

The diversity of responses mediated by individual S1P receptors begins with the immediate downstream signaling partners: the heterotrimeric G proteins. As GPCRs, S1P receptors rely on heterotrimeric G proteins to mediate downstream signaling. The five families of heterotrimeric G proteins all have different downstream signaling

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components and cellular effects. Each S1P receptor isoform couples only with specific G protein families (Figure 1.3). Therefore, specific G protein couplings give unique signaling profiles to each S1P receptor.

$S1P_1$, for example, couples only to G_i family G proteins (Figure 1.3A; Windh et al., 1999), leading to inhibition of adenylyl cyclase and decreases in intracellular cyclic adenosine monophosphate (cAMP) (Okamoto et al., 1998; Van Brocklyn et al., 1998; Zondag et al., 1998). Another affect of $S1P_1$ signaling is the activation of phospholipase C (PLC) (Okamoto et al., 1998). This PLC activation leads to stereotypical Ca^{2+} mobilization in cells with activated $S1P_1$ receptor. In addition to the cAMP and Ca^{2+} responses, $S1P_1$ can activate the Map kinases ERK1 and 2. ERK activation by $S1P_1$ was shown to be dependent on phosphoinositol 3-kinase (PI3K). Activation of PI3K is not downstream of signaling by the G protein α subunit, but relies on the activity of the $G\beta\gamma_i$ dimer (Pyne and Pyne, 2000). Finally, signaling downstream of $S1P_1$ is a potent activator of the small GTPase Rac (Okamoto et al., 2000), also downstream of PI3K.

In contrast to $S1P_1$, $S1P_2$ couples to three G protein families (Figure 1.3B): G_i, G_q and G_{12}. As would be expected, $S1P_2$ can also activate PLC and Ca^{2+} mobilization in cells. However, unlike $S1P_1$, the Ca^{2+} mobilization downstream of $S1P_2$ is only partially inhibited by pertussis toxin, an inhibitor of G_i signaling (Kon et al., 1999). The fact that this Ca^{2+} mobilization is only partially inhibited is likely due signaling downstream of $S1P_2$ mediated by G_q family G proteins, which are also known to mobilize Ca^{2+} in a PLC dependent fashion. $S1P_2$ is also capable of activating the ERK1/2 MAPK pathways and, like $S1P_1$ activation of ERK, this activation appears to be mediated via G_i (Okamoto et al., 1999).

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Figure 1.3

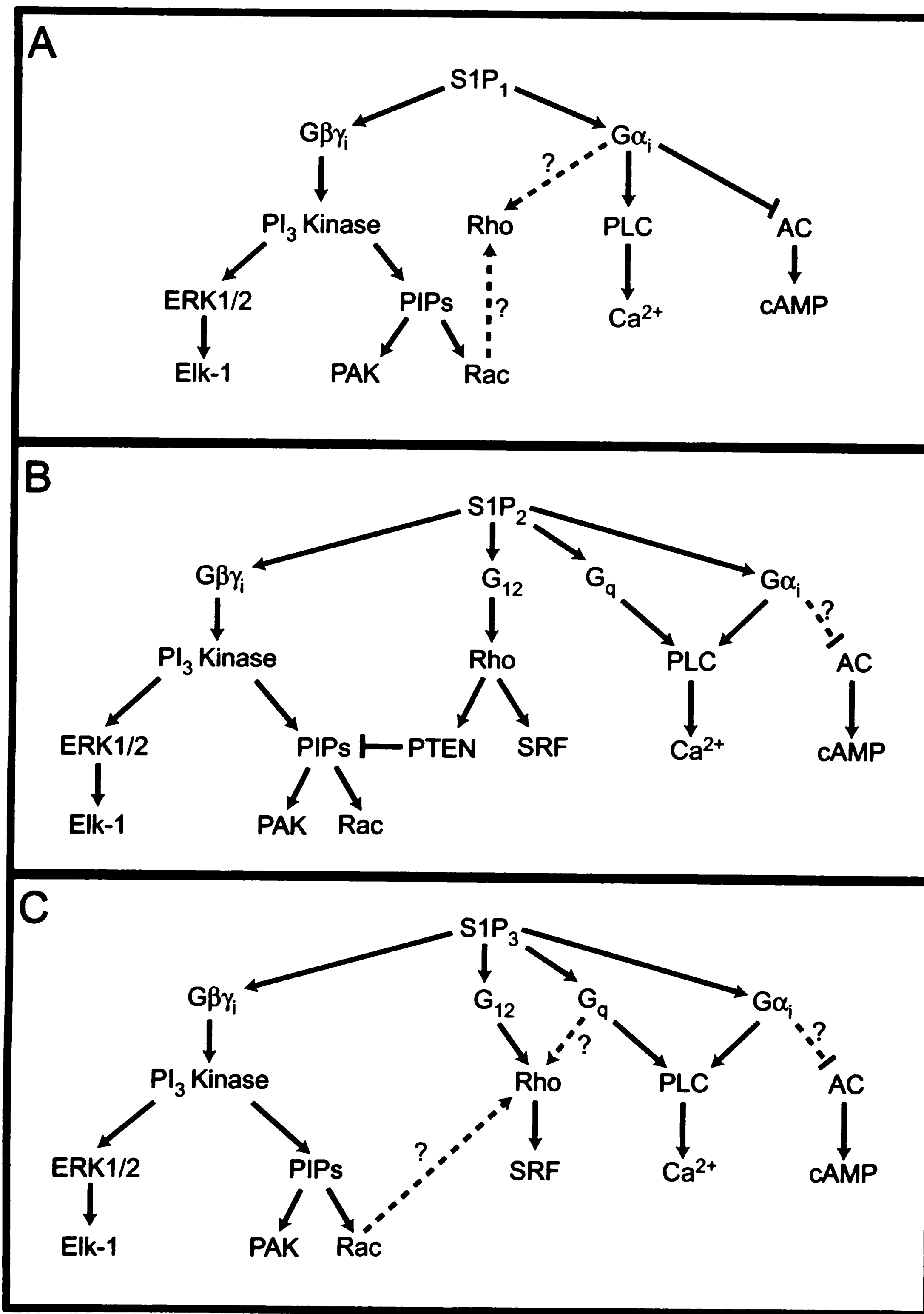

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Figure 1.3: Signaling downstream of $S1P_1$, $S1P_2$ and $S1P_3$

(A) Downstream signaling network upon binding of S1P to the mammalian $S1P_1$ isoform.

(B) Downstream signaling network upon binding of S1P to the mammalian $S1P_2$ isoform.

(C) Downstream signaling network upon binding of S1P to the mammalian $S1P_3$ isoform.

Solid arrows (→) indicate known activation steps. Solid lines ending with bars (⊣) indicate known inihibition steps. Dashed lines (?) indicate putative activation or inhibition steps inferred from responses of other receptors or receptor families. AC = adenylate cyclase; cAMP = cyclic adenosine monophosphate; ERK = extracellular regulated kinase; PI_3 Kinase = phosphoinositol-3 kinase; PIPs = phosphoinositol phosphates; PLC = phospholipase C; PAK = p21 activated kinase; SRF = serum response factor.

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Although G_i and G_q signaling are activated by $S1P_2$, many of the key effects of $S1P_2$ appear to be mediated downstream of the G_{12} family of G proteins. These G proteins have been shown to mediate much of their signaling via the small GTPase Rho (reviewed in Riobo and Manning, 2005). It was noted in cell culture experiments, that activation of $S1P_2$ results in stress fiber formation (Gonda et al., 1999), a common effect of Rho mediated signaling. Subsequently, it has been shown that Rho activation downstream of $S1P_2$ mediates a great deal of the receptor's unique signaling effects. Specifically, it was shown that Rho activation by $S1P_2$ results in inhibition of the related small GTPase Rac and the kinase PAK (Gonda et al., 1999). This antagonism is controlled by PTEN (Sanchez et al., 2005), a phosphatase that antagonizes the activity of PI3K by dephosphorylating phosphatidyl inositol phosphate products (PIP). Rac and PAK are two key regulators of cell motility and PI3K is the upstream activator of both Rac and PAK via PIP products.

Signaling via $S1P_3$ shares some similarity to that of $S1P_2$. Like $S1P_2$, $S1P_3$ couples to G_i, G_q, and G_{12} family members (Figure 1.3C). As with $S1P_2$, $S1P_3$ can activate PLC activity and Ca^{2+} responses in a manner only partially sensitive to PTX (Kon et al., 1999; Okamoto et al., 1999). In addition, $S1P_3$ activates ERK1/2 signaling in in a $G\beta\gamma$ dependent fashion (Okamoto et al., 1999). In cells expressing $S1P_3$, S1P is also capable of causing stress fiber formation in a Rho dependent fashion (Lee et al., 1999a). This Rho mediated signaling is likely to be dependent on signaling via the G_{12} and possibly G_q families of G proteins. However, unlike $S1P_2$, $S1P_3$ does not inhibit the activities of the small GTPase Rac. In fact, signaling via $S1P_3$ activates the Rac pathway (Okamoto et al., 2000).

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With $S1P_2$ and $S1P_3$ coupling to the same downstream G proteins it is difficult to reconcile the fact that these receptors have differing capacities to activate or inhibit Rac. The answer to this dilemma, it seems, has to do with the level of signaling via G_{12} versus G_i family members. It has been shown that blocking $S1P_2$ mediated G_{12} activation disrupts the ability of this receptor to inhibit Rac activity (Sugimoto et al., 2003). Unexpectedly, it was found that blocking $S1P_3$ mediated G_i signaling and presumably increasing G_{12} activation change $S1P_3$ such that it actively inhibits Rac activation. These results suggest that cells integrate the counteracting signals coming from G_{12} and G_i. In the case of $S1P_2$, the G_{12} mediated inhibition of Rac is dominant; while in the case of $S1P_3$ G_i signaling dominates, leading to activation of Rac.

As this section has discussed, the S1P specific GPCRs are capable of eliciting responses via many downstream signaling cascades. However, examination of such signaling responses individually does not give a complete appreciation of the actual effects of signaling via S1P receptors. After all, these cellular signals do not occur in a vacuum, but interact with one another and with unrelated cellular signals to give complex cellular outputs. In the next section I discuss some of the known effects $S1P_1$, $S1P_2$ and $S1P_3$ have on the behaviors of actual cells.

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1.VI. Cellular effects of sphingosine 1-phosphate receptor signaling

Two of the most studied effects of signaling via the EDG family of receptors are their effects on cell proliferation and cell migration. These two effects are interesting in the case of the S1P receptors, because they highlight the importance of the individual receptor subtypes in generating unique cellular responses. All three of the S1P receptors

in discussion, $S1P_1$, $S1P_2$ and $S1P_3$, affect both of these processes in unique ways. The examples presented here are selected because they show these unique contributions by individual S1P receptors to cell proliferation and migration. The different effects of each receptor give S1P the potential to generate different effects on many cellular responses depending on the repertoire of S1P receptors present.

1.VIa. Sphingosine 1-phosphate receptors in cell proliferation

It has been well documented that sphingosine 1-phosphate (S1P) is a potent regulator of cell proliferation (Zhang et al., 1991). However, one uncertainty that plagued the question of S1P's role in cell proliferation was the relative contribution of individual S1P receptor subtypes in stimulating cell proliferation.

The first receptor isoforms tested for their capacity to induce cell proliferation were $S1P_2$ and $S1P_3$. In a rat hepatoma cell line both $S1P_2$ and $S1P_3$ caused cells to increase their rate of DNA synthesis in response to exogenously applied S1P (An et al., 2000). In addition, both $S1P_2$ and $S1P_3$ activated *AP-1*, *c-fos*, *c-jun*, Erk dependent Elk-1 and Rho dependent serum response factor (SRF) transcription in response to S1P. Upregulation of transcription by these factors are indicative of cells in a proliferative state.

In a study investigating the role of S1P receptors on the proliferation of vascular smooth muscle cells (VSMCs), $S1P_1$ was shown to have a different mechanism for activating cell proliferation than $S1P_2$ and $S1P_3$ (Kluk and Hla, 2001). Kluk and Hla first examined the expression of S1P receptor isoforms in two different rat VSMC populations. The relatively immature pup-intimal VSMCs express all three of the major

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S1P receptors, $S1P_1$, $S1P_2$ and $S1P_3$. In contrast, the more mature adult-medial VSMCs lack expression of $S1P_1$ and only express $S1P_2$ and $S1P_3$.

The baseline proliferation of pup-intimal and adult-medial VSMCs are quite different from one another, with the pup-intimal VSMCs having a much higher rate of proliferation in the absence of exogenous S1P. Both cell types, however, responded to S1P addition by increasing their DNA synthesis and proliferation. The responses the two cell types had to exogenous S1P addition were not identical, however. For instance, pup-intimal VSMC proliferative response to S1P was inhibited by the addition of PTX. In contrast, adult-medial VSMC proliferation was unaffected by PTX. Interestingly, inducing $S1P_1$ expression in the adult-medial VSMCs, however, converted this PTX-insensitive proliferation to an entirely PTX-sensitive response. This finding was striking, because it suggests that the addition of a single S1P receptor isoform can fundamentally change the nature of a cell's response to S1P, even if that cell already expresses other S1P receptors. These data also suggest there may be a hierarchy of signaling by S1P receptors in cells with multiple receptor isoforms, such that one receptor sub-type is capable of dominating over the signaling of other receptor isoforms.

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1.VIb. Sphingosine 1-phosphate receptors in cell migration

Another well studied cellular process potently affected by S1P receptors is that of cell migration. S1P has been shown in several cell types to be an activator of chemotaxis, or directed cell migration. Much of the understanding of the role of S1P and other lipids in cell migration comes from the study of cells of the cardiovascular system. Several lipid signaling mediators, including S1P, LPA, phosphatidic acid and

sphingosylphosphorylcholine, can direct cell migration in endothelial and vascular smooth muscle cell (VSMC) populations (Boguslawski et al., 2000; English et al., 1999; Kluk and Hla, 2001; Lee et al., 2000; Lee et al., 1996; Lee et al., 1999b; Panetti et al., 2000). Of these lipids, S1P seems to be the most potent mediator of chemotaxis.

It is important to note that not all of the three major S1P receptors expressed in vascular cell populations, $S1P_1$, $S1P_2$ and $S1P_3$, are sufficient for mediating cell migration. In fact, only $S1P_1$ and $S1P_3$ have been implicated in the positive regulation of cell migration (Paik et al., 2001; Wang et al., 1999). $S1P_1$ is the most efficient regulator of chemotaxis in response to S1P exposure, while $S1P_3$, in contrast, is less effective in directing cell migration. Both $S1P_1$ and $S1P_3$ activate the small GTPase Rac in response to S1P exposure. This correlation between chemotaxis and Rac activation is not surprising as Rac is a known regulator of lamellapodia formation and protrusive membrane activity in response to chemotactic factors (Ridley et al., 1992). These protrusive cell behaviors are necessary for guided cell migration and in the absence of Rac function cells fail to migrate.

Phenotypes of the mouse *S1p1* knockout animal suggested that S1P might also have roles in cell migration in response to certain chemotactic growth factors. Several groups have investigated whether or not S1P mediated signaling might affect PDGF directed chemotaxis, based on the knowledge that PDGF is an activator of SK activity (Olivera and Spiegel, 1993). Some studies have suggested S1P and signaling via $S1P_1$ are required for PDGF mediated chemotaxis (Hobson et al., 2001; Liu et al., 2000; Rosenfeldt et al., 2001). However, the idea that $S1P_1$ is required to mediate PDGF mediated chemotaxis is currently in contention, as work by another group has suggested

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that the requirement for $S1P_1$ in PDGF mediated chemotaxis may not be an absolute (Kluk et al., 2003). In contrast to earlier studies, this study has shown that mouse embryonic fibroblasts and VSMCs lacking *S1p1* migrate normally in response to PDGF exposure. The discrepancies in these studies have not been truly resolved and may require further examination. It is, however, interesting that activation of SK by growth factors might lead to autocrine activation of $S1P_1$ and $S1P_3$ in cells receiving the growth factor signaling. This autocrine signaling might then be a contributing factor in chemotactic responses to growth factor signaling.

Thus far I have focused on the positive role that S1P has in chemotactic cellular responses. However, not all signaling via S1P receptors has positive effects on chemotaxis. As stated above, $S1P_1$ and $S1P_3$ both activate the small GTPase Rac, a known requirement for chemotaxis. What then is the effect on chemotaxis of $S1P_2$ signaling which actively inhibits Rac? As one would expect $S1P_2$ is an anti-chemotactic receptor. The role of S1P signaling as an inhibitor of cell migration was first demonstrated in B16 melanoma cell lines (Sadahira et al., 1992). In initial studies the defect in cell migration was attributed to an overproduction of focal adhesions and an inhibition of protrusive activity in response to Rho activation by S1P mediated signaling (Yamamura et al., 1996; Yamamura et al., 1997). Later the mechanism of this chemotactic inhibition was shown to be the active inhibition of Rac activity by $S1P_2$ (Okamoto et al., 2000). It has since been shown that the inhibition of chemotaxis by $S1P_2$ plays a role in blocking PDGF mediated cell migration and proliferation in cells expressing both the PDGF receptor and $S1P_2$ (Goparaju et al., 2005). Therefore,

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although the positive relationship between $S1P_1$ and PDGF mediated chemotaxis remains in doubt, there is evidence for a negative relationship between $S1P_2$ and PDGF signaling.

All experimental evidence suggests that $S1P_1$ and $S1P_3$ are positive regulators of chemotaxis while $S1P_2$ actively inhibits cell migration. The opposing behaviors of $S1P_1$ and $S1P_2$ on migration begs the question what is the effect of S1P exposure on cells expressing both of these S1P receptor subtypes? This question illustrates the complexity of signaling via S1P receptors and generally the answer, in investigated cases, has depended on the relative expression levels of each receptor subtype. As expected, cell types that are known to actively migrate towards S1P sources, such as human umbilical vascular endothelial cells (HUVECs), bovine pulmonary artery endothelial cells and rat pup-intimal VSMCs, have relatively high levels of expression of the $S1P_1$ receptor isoform (Kluk and Hla, 2001; Wang et al., 1999). In contrast, rat aortic and medial endothelial cells express little $S1P_1$, have high levels of $S1P_2$ and fail to initiate migratory responses towards S1P sources (Kluk and Hla, 2001). The ability of cell types such as VSMCs to change their migratory responses to S1P by changing receptor sub-type expression patterns suggests a mechanism by which cells at different developmental or physiological states might have vastly different responses to S1P (Figure 1.4). This dynamic responsiveness to S1P, again, makes this molecule a flexible signaling factor.

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1.VII. Sphingosine 1-phosphate signaling in development and adult physiology

The question I now deal with is how these signaling and cellular effects of S1P actually impact a living organism. Specifically, I discuss the roles S1P has in the development and function of the vertebrate cardiovascular system and the emerging understanding of

Figure 1.4

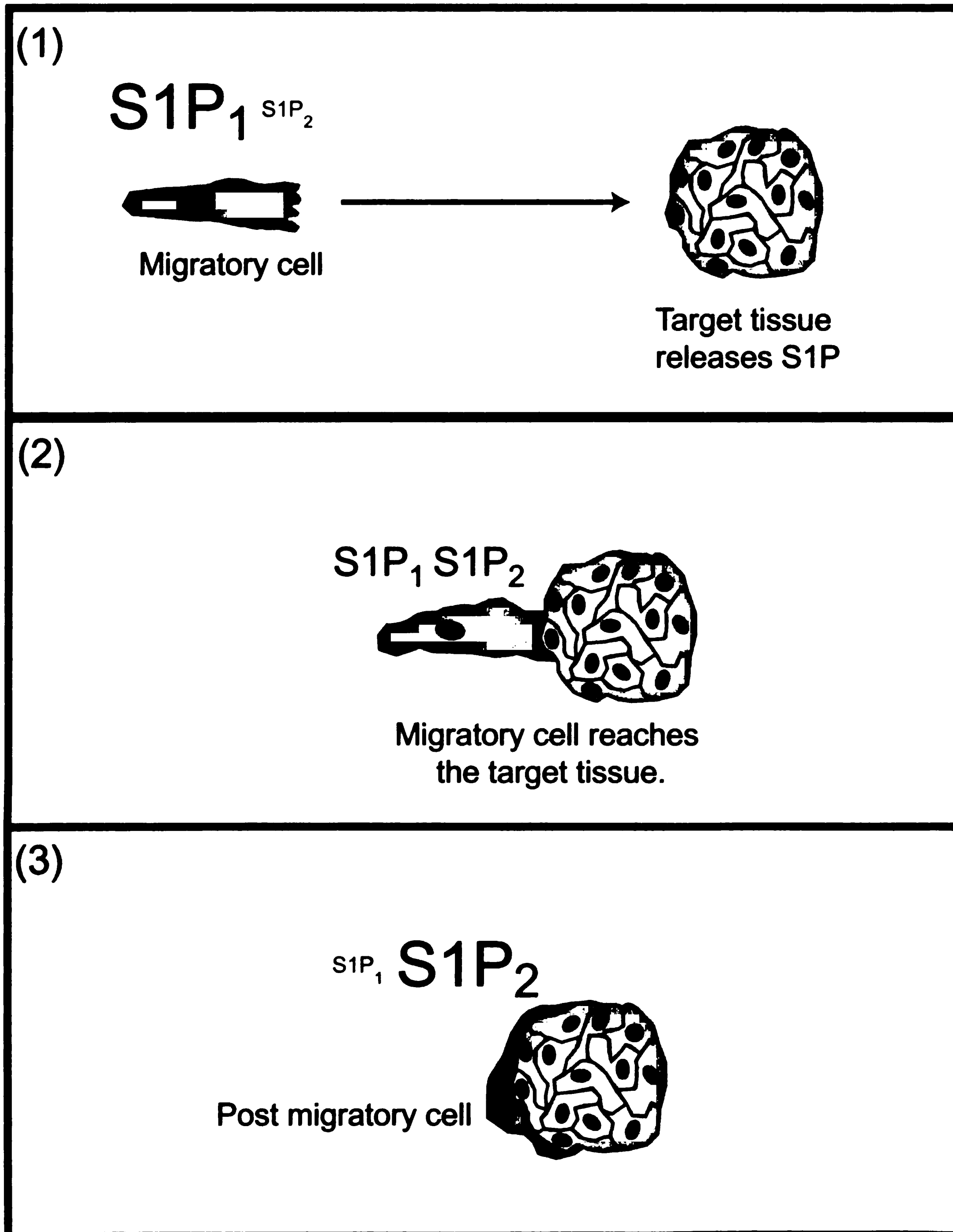

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Figure 1.4: Receptor isoform switching

The migratory cell (green) in panel (1) expresses high levels of $S1P_1$, a pro-migratory S1P receptor, and low levels of $S1P_2$, the anti-migratory S1P receptor. In response to the release of S1P by the target tissue (red), the migratory cell moves to the target tissue (arrow). When the migratory cell reaches the target tissue in panel (2), downregulates $S1P_1$ and upregulates $S1P_2$. In panel (3), the cell becomes post migratory and S1P inhibits chemotaxis due to high $S1P_2$ and low $S1P_1$ expression. Thus S1P signaling keeps the cell associated with the target tissue.

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how S1P impacts the function of the immune system. These two areas are not the only organ systems and tissues affected by S1P signaling; however, these areas of study are excellent for examining the interplay of the S1P receptor isoforms in the living organism.

1.VIIa. Sphingosine 1-phosphate in the development of the cardiovascular system

The first evidence of a developmental role for S1P receptors came from studies we carried out in the zebrafish, utilizing the *miles apart* mutant. I discuss the nature of this mutation and recent advancements in our understanding of how this gene affects heart development in zebrafish in greater detail in the second chapter of this dissertation. However, I would like to briefly contrast the effects of loss of function of the *miles apart/s1p2* genes in zebrafish and mouse.

In zebrafish embryos, loss of *mil* gene function results in a striking defect in heart development. The primitive heart tube in zebrafish, as in all vertebrates, is formed by the fusion of two fields of precardiac mesoderm (PCM) that arise in the anterior lateral plate mesoderm (see Figure 2.1). In *mil* mutants these PCM precursors fail to migrate to the embryonic mid-line and never fuse to form the primitive heart tube (Figure 2.1C; Kupperman et al., 2000). As a result of this failure in precardiac mesoderm fusion, *mil* mutant embryos develop bilateral heart structures, a condition called cardia bifida. The heart-like structures in *mil* mutants are unable to support normal circulation and the result is embryonic lethality.

In contrast to the zebrafish *mil* mutant embryos, the phenotypes seen in mouse embryos lacking the *S1p2* gene have milder effects (MacLennan et al., 2001). *S1p2*$^{-/-}$ mouse embryos have normal heart development and show no signs of vascular defects

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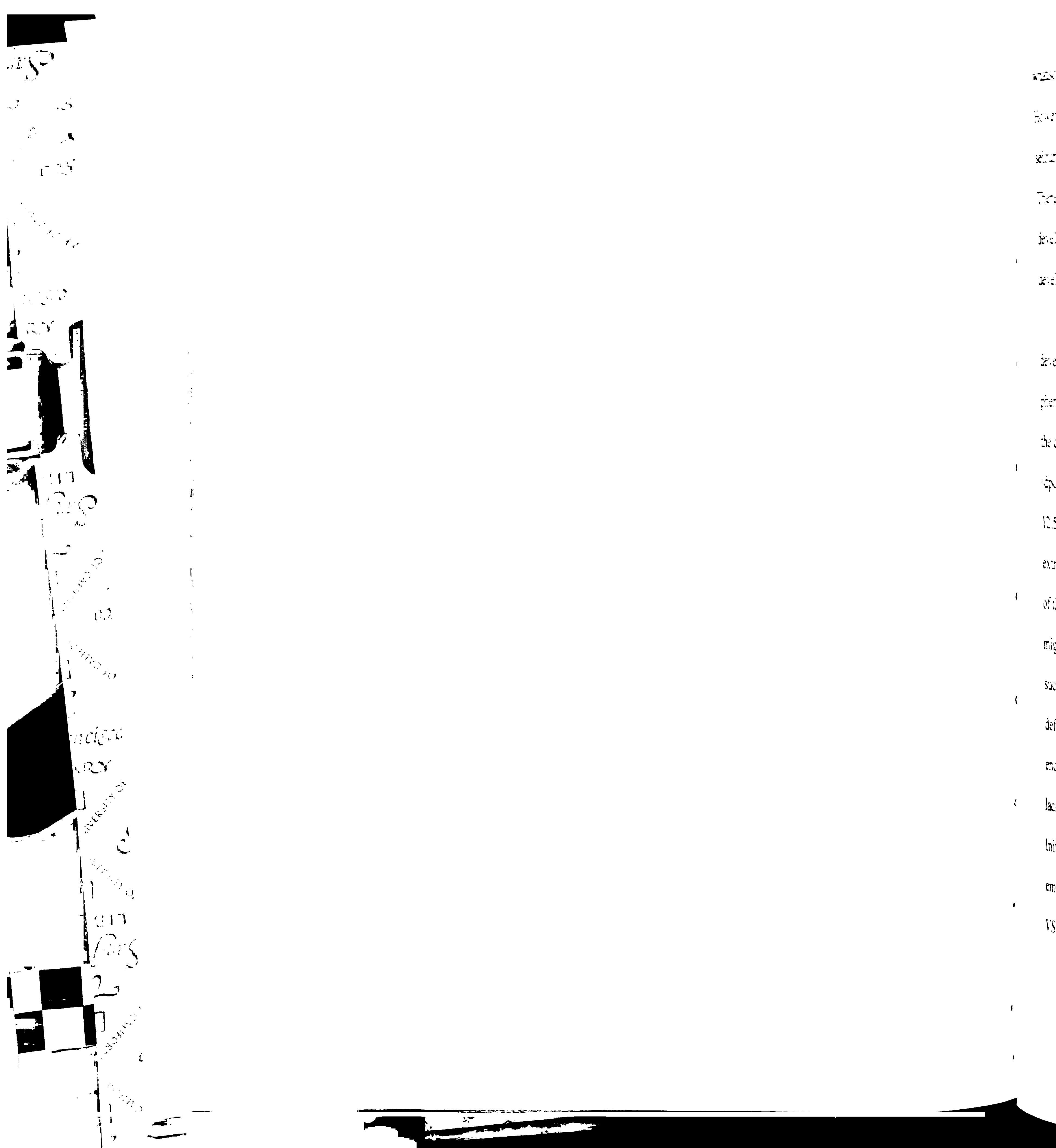

whatsoever. Instead *S1p2* mutant mice survive past birth and are superficially normal. However, as they age these mice begin to develop spontaneous and sometimes fatal seizures. In addition, they display increased neuroexcitability in some neuronal types. Therefore, although the zebrafish $S1P_2$ orthologue is critical for normal heart development, the mouse $S1P_2$ receptor is seemingly less important in cardiovascular development and instead plays a role in the function of the central nervous system.

The *S1p1* gene is also expressed in the mammalian cardiovascular system during development. Loss of this receptor, therefore, might be expected to give heart or vascular phenotypes in mammalian systems. In the case of the murine *S1p1* gene this is, in fact, the case. Mice lacking *S1p1* gene function do not survive beyond 14. 5 days post coitus (dpc) due to defects in the cardiovascular system (Liu et al., 2000). Beginning as early as 12.5 dpc, *S1p1*$^{-/-}$ embryos show signs of severe hemorrhaging in both embryonic and extraembryonic vasculature. This hemorrhaging is the result of defects in the maturation of the vasculature in *S1p1*$^{-/-}$ embryos. In wild-type embryos the VSMC population migrates around the endothelium from the ventral toward the dorsal side of blood vessels such as the dorsal aorta, in order to form the vessel wall (Figure 1.5A). This process is defective in *S1p1* knockout mice (Figure 1.5B). As a result of the failure in VSMC enclosure of blood vessels, the endothelial cells in *S1p1* null mice are unsupported. This lack of support results in breach of the integrity of the blood vessels and hemorrhaging. Initially it was unclear whether the defects seen in vascular maturation in *S1p1* mutant embryos were due to a lack of $S1P_1$ mediated signaling in the endothelium or in the VSMCs, as both of these cell types express *S1p1* (Liu et al., 2000).

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Using Cre mediated recombination technology the *S1p1* gene was specifically deleted in the endothelial cell lineage in order to ask the question of whether or not the receptor functions in the endothelium (Chae et al., 2004). Mice lacking *S1p1* specifically in endothelial cells look similar to mice lacking *S1p1* in all tissues. Therefore, *S1p1* function in the endothelium is necessary for normal maturation of the vasculature. Further study has shown that this necessity for *S1p1* in endothelial cells is due to the important role this receptor has in regulating N-cadherin (Figure 1.5C and D; Paik et al., 2004). In *S1p1* mutant embryos, N-cadherin is not localized appropriately to cell-cell contacts and potentially leads to defects in endothelial cell adhesion to other endothelial cells and to VSMCs. Loss of N-cadherin expression in the endothelium is also sufficient to cause defects in VSMC migration around blood vessels (Figure 1.5E), suggesting that N-cadherin regulation by $S1P_1$ is required for vascular maturation (Paik et al., 2004).

Interestingly investigations using mice lacking multiple S1P receptor isoforms have also turned up unexpected effects on vascular development. *S1p2* knockout mice have no obvious defects in the establishment of normal blood vessels, as was discussed earlier. However, mice that lack both the *S1p1* and *S1p2* genes show an earlier onset and increased severity of hemorrhaging as compared to *S1p1* single knockout mice (Kono et al., 2004). The observation that loss of $S1P_2$ mediated signaling increases the vascular defects seen in *S1p*$^{-/-}$ embryos suggests that the $S1P_1$ and $S1P_2$ receptors are acting in concert during the formation of blood vessel walls. Given the data presented earlier in this thesis with regards to the antagonistic relationship between these two receptors in cell migration their apparent cooperative behavior in vascular maturation is somewhat of a surprise. However, the analysis of this interaction is in its infancy and it is not yet clear

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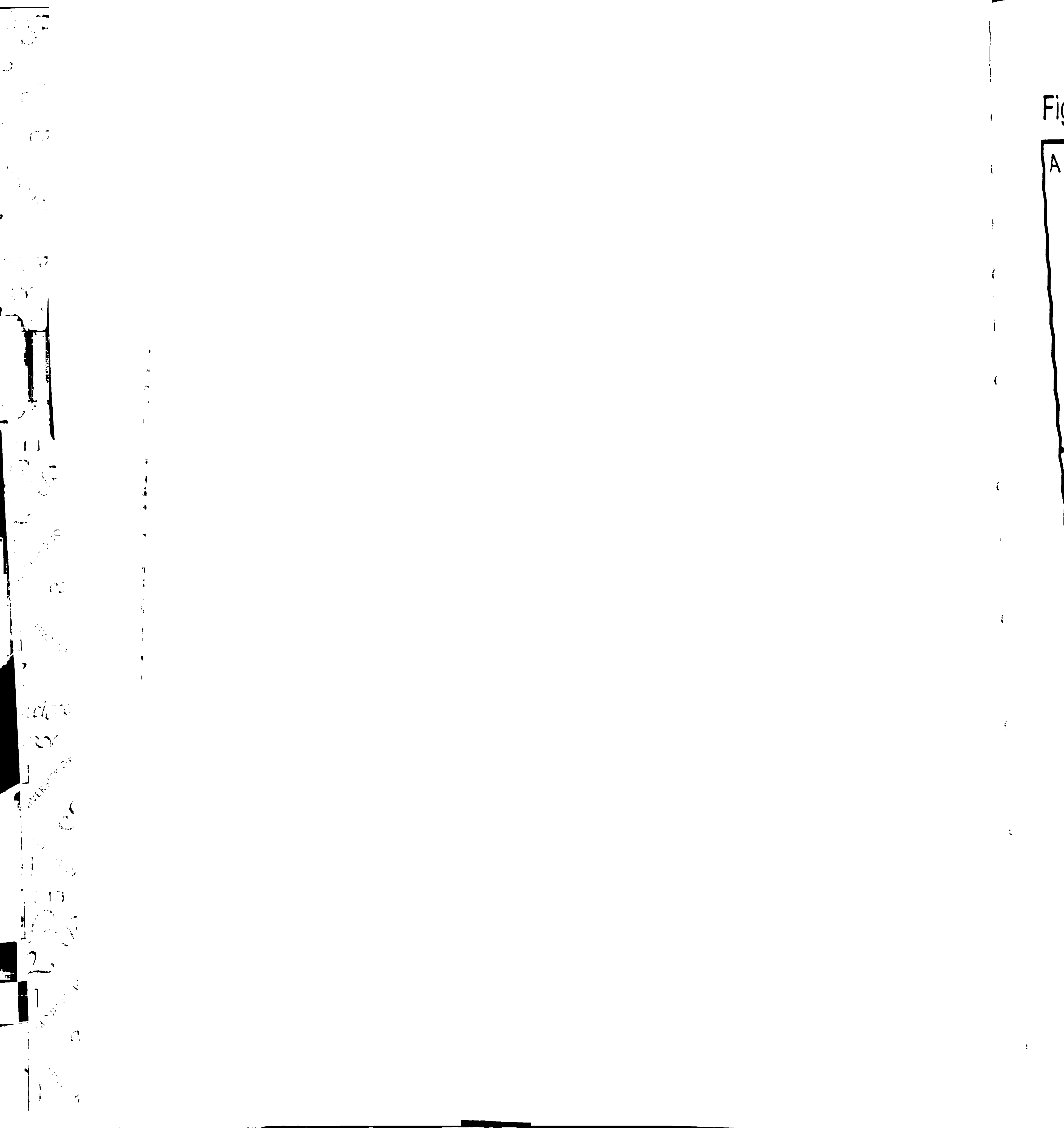

Figure 1.5

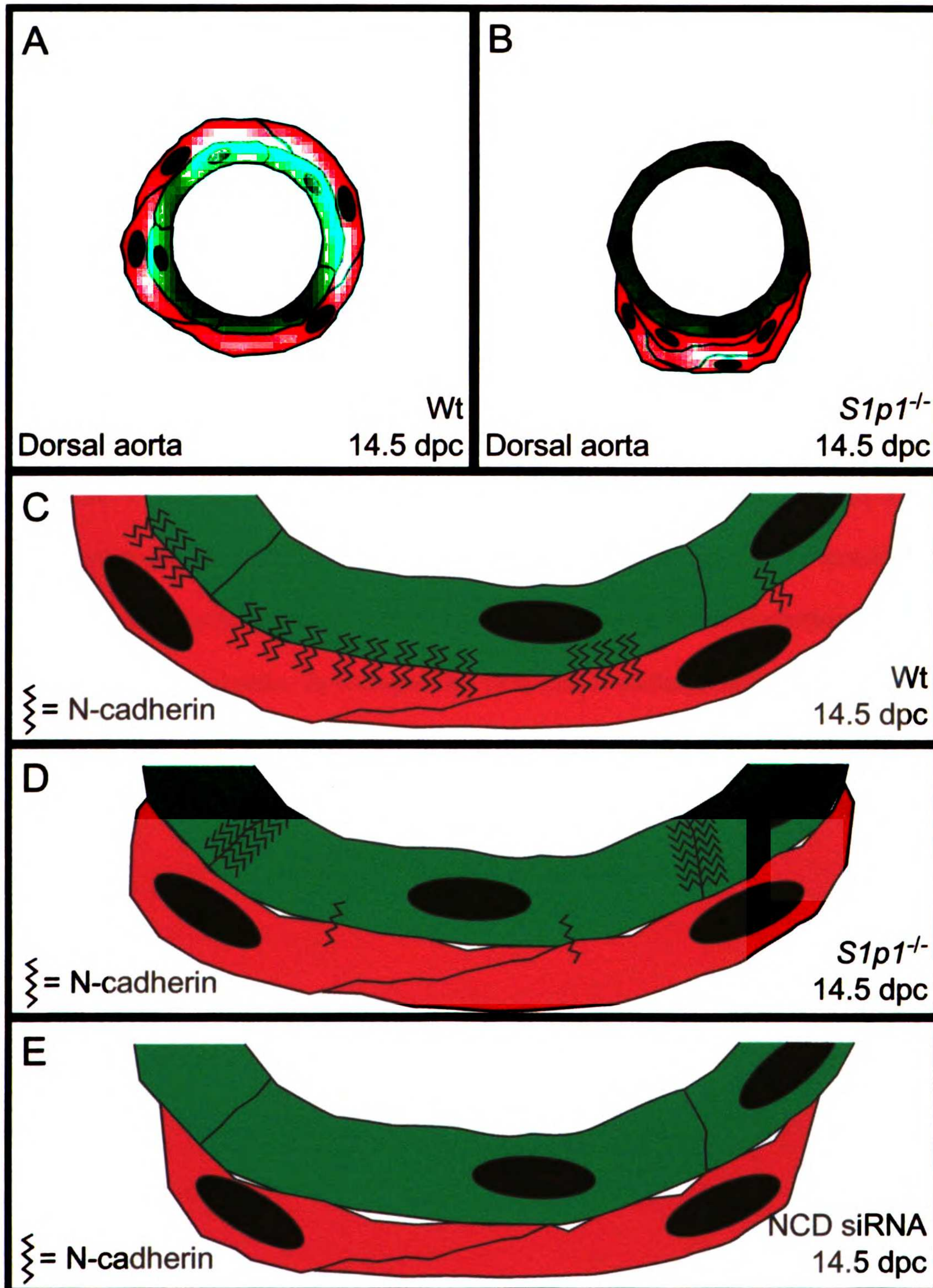

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Figure 1.5: $S1P_1$ and vascular maturation

(A and B) Schematized crossections of mouse embryonic dorsal aorta at 14.5 dpc. In wild-type embryos (A), VSMCs (red) have entirely surrounded the endothelial cell tube (green) at 14.5 dpc. In *S1p1*$^{-/-}$ embryos (B), the VSMCs fail to migrate from their initial ventral positions and do not surround the endothelial tube. This failure in mural investment with VSMCs leads to rupture of the blood vessel walls. (C,D and E) Blowups showing schematized representations of the interaction between VSMCs (red) and endothelial cells (green). In wild-type embryos at 14.5 dpc the VSMCs and endothelial cells show high levels of N-cadherin staining between them. In *S1p1*$^{-/-}$ embryos, by contrast, the N-cadherin is oriented to the junctions between endothelial cells and is not found between endothelial cells and VSMCs. These VSMCs fail to migrate around the endothelial cells. The migration of VSMCs is dependent upon the reorientation of N-cadherin in endothelial cells. In embryos that have had N-cadherin specifically knocked down using small interfering RNA (D) there is no N-cadherin mediated adhesion between VSMCs and endothelial cells and VMSCs also fail to migrate dorsally around the endothelium.

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whether $S1P_1$ and $S1P_2$ are signaling within the same cell. $S1P_1$ signaling is clearly important in the endothelial cells of the vasculature, so perhaps $S1P_2$ signaling is acting in the VSMCs during the process of mural investment of blood vessels. It remains to be seen, however, if this explanation is correct or if the regulation of VSMC migration by these receptors is more complex.

Finally, it should be noted that while *S1p1* and *S1p2* genes are functioning in vascular maturation, the role of *S1p3* in this process, is unclear. *S1p3* knockout mice, as is seen with *S1p2* mutants, have no detectable defects in the vasculature or heart (Ishii et al., 2001). In fact, these mice show no overt phenotypic effects due to loss of *S1p3*. When *S1p3* mutations are combined with *S1p1* mutations there are no additional effects on the vascular phenotype (Kono et al., 2004). There is some suggestion from the analysis of mice lacking all three of the major S1P receptors, though, that *S1p3* might increase the severity of the vascular phenotypes over that seen in *S1p1*$^{-/-}$; *S1p2*$^{-/-}$ mice (Kono et al., 2004). However, the authors of this study themselves point out that the numbers of triple mutant mice analyzed are so low that these experiments may not be statistically interpretable (Kono et al., 2004).

As one can see S1P and its receptors are important regulators of development of the cardiovascular system in at least two organisms. Because of the effects on the development of the cardiovascular system by S1P receptor function, one might be curious as to whether or not these receptors also are involved in the physiology of the mature cardiovascular system. In the next section I discuss some of what is known about the role S1P and its receptors may be playing in the cardiovascular system later in life.

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1.VIIb effects of S1P on cardiovascular physiology and function

As was discussed in the previous section, S1P and its receptors have critical roles in the establishment of the cardiovascular system. Once established, however, these receptors continue to play roles in the function of the heart and blood vessels. Unfortunately there is less understanding about the roles of individual receptors later in life. It is clear, however, that S1P can affect several key functions in the cardiovascular system, including regulation of vascular tone, the ischemic response and cardiac rhythm. These effects and others will be discussed below.

Several studies have looked into the effects of S1P exposure on venous and arterial tone. In several studies using cultured rat blood vessels it has been shown that exposure to S1P leads to potent vasoconstriction (reviewed in Alewijnse et al., 2004). It has also been shown that systemic injection of S1P into rats caused a significant decrease in blood flow to the intestines and through the kidneys, presumably due to the proposed vasoconstrictive effects of S1P (Bischoff et al., 2000a; Bischoff et al., 2000b). Little is known, however, about which receptor isoforms regulate this response of the vasculature to acute S1P exposure. The effects are known to be PTX sensitive, suggesting the involvement of G_i coupled receptors (Bischoff et al., 2000b). Also given the fact that the vascular smooth muscle is likely to be the affected target causing vasoconstriction, one could speculate that either $S1P_2$, $S1P_3$ or both of these receptors are involved, as they are the predominant S1P receptor subtypes expressed in mature VSMCs (Coussin et al., 2002). The idea that $S1P_3$ is involved in this process has been supported by a study using knockdown of the *S1p3* gene as well as a pharmacological agent affecting $S1P_3$ function (Salomone et al., 2003). It is not clear, however, what the contribution of $S1P_1$ and $S1P_2$

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might be in vasoconstriction, although in explanted coronary artery tissue explants $S1P_2$ mediated signaling can cause VSMC contraction (Ohmori et al., 2003).

In addition to confusion over the relative contribution of receptor isoforms to the effect of S1P on vasoconstriction it is also not clear whether the effect of S1P is, indeed, solely on the VSMC. In one study examining the effect of S1P exposure on vasoconstriction, S1P exposure actually caused vasodilation (Mogami et al., 1999). Vasodilation in response to S1P was dependent on the presence of endothelial cells and on nitric oxide synthesis. This study exemplifies the fact that the effect of S1P on blood vessel tone is somewhat complicated. Furthermore, this study shows that S1P signaling cannot be thought of as a process occurring in a vacuum, as other signaling molecules modulate S1P signaling output. Regardless of the exact receptor isoform or isoforms involved it is clear that S1P can affect the state of vascular constriction in living animals.

One of the most intriguing discoveries surrounding S1P recently is the finding that S1P is a cardioprotective factor in cases of ischemic injury (reviewed in Karliner, 2004). The first evidence for S1P having a cardioprotective effect was discovered when exposure of cardiomyocytes to S1P in cell culture was shown to block cell death in response to hypoxia (Karliner et al., 2001). Furthermore, it was shown that the effects of other cardioprotective agents such as TNFα and GM-1 monoganglioside are mediated through activation of SK (Jin et al., 2004; Lecour et al., 2002). Finally, studies have shown that level of SK expression in the heart has an inverse relationship to infarct size in mice treated with cardiac ischemia/reperfusion injury (Jin et al., 2004). Very little data exist to suggest how S1P signaling is involved in preconditioning myocardium against death and contractile defects after ischemic challenge. However, given the importance of

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heart disease to the medical community this is likely to be an eagerly pursued topic as it may lead to novel treatments in patients at risk for or who have suffered myocardial infarction.

Another important cardiac process mediate in part by S1P is the maintenance of heart rate. One of the known effects seen in humans treated with the S1P receptor agonist, FTY720, is bradycardia, or reduced heart rate (Budde et al., 2002). Although a relatively minor side effect of FTY720 treatment, bradycardia does suggest that S1P is capable of modulating mammalian heart rate. A subsequent study sought to isolate the S1P receptor responsible for decreasing heart rate in FTY720 and S1P treated mice. This study discovered that while the beneficial immunosuppressant characteristics of FTY720, discussed later, were the result of $S1P_1$ signaling, the modulation of heart rate was regulated by $S1P_3$ (Sanna et al., 2004). Fortuitously, the decrease heart rate is only a transient response to FTY720 treatment that disappears after prolonged exposure to the drug (Budde et al., 2002; Sanna et al., 2004).

As I have discussed in this section S1P signaling plays critical roles in the function of the mammalian cardiovascular system. As a result of these and other roles, S1P signaling has become an important focus for clinical research. Although the use of S1P signaling modulators in the treatment of cardiovascular pathology has not reached the level seen in regulation of the immune system, it is clear that S1P signaling may have important contributions to cardiovascular medicine.

1.VIII. Sphingosine 1-phosphate and the immune system

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Cardiovascular biology is not the only discipline that has investigated the function of S1P signaling in the living organism. Although many of the phenotypes uncovered by knockout of the receptors have revolved around vascular biology, S1P also modulates the immune system. In this section I discuss the prominent roles S1P signaling has been shown to have in the trafficking and development of lymphocytes.

1.VIIIa. Sphingosine 1-phosphate in lymphocyte trafficking

As was mentioned before the S1P receptor agonist FTY720 has emerged as an important pharmaceutical agent. FTY720, a sphingosine analogue, was isolated in a screen looking at derivatives of the compound myriocin (Fujita et al., 1996). In the initial study, Fujita et al. were able to demonstrate that FTY720 was an immunosuppressive agent that was effective in the prevention of tissue rejection in rat skin allograft assays. A great deal of excitement surrounded this discovery as FTY720 represented a class of novel, safe immunosuppressants. As was mentioned in the previous section discussing the role of S1P in regulating heart rate, FTY720 does cause minor bradycardia that self corrects after prolonged exposure. However, in comparison to the typical cyclosporine based immune suppression this side effect is quite minor (reviewed in ten Berge and Schellekens, 1994).

FTY720 was shown to be a selective agonist for $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$, but not $S1P_2$ (Brinkmann et al., 2002; Mandala et al., 2002). However, unmodified FTY720 does not act on S1P receptors itself. Instead, the compound is phosphorylated by SK2, as was previously mentioned, and the phosphorylated form (FTY720-P) acts on the receptors as an S1P analogue (Sanchez et al., 2003).

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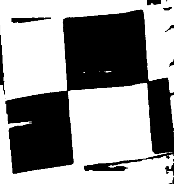
FTY720-P exposure suppresses the immune response by causing rapid and prolonged lymphopenia, or absence of lymphocytes in the blood. This lymphopenia is the result of lymphocyte populations accumulating in the lymphoid organs (Chiba et al., 1998). The knowledge that FTY720-P exposure leads to the sequestration of lymphocytes in secondary lymphoid organs via S1P receptor activation led to the question of which receptors might be involved in this process. T and B cells primarily express $S1P_1$ and $S1P_4$ isoforms of S1P receptors (Graeler and Goetzl, 2002), suggesting that these may be the S1P receptors modulating lymphocyte localization. Studies into this question have demonstrated that the critical receptor isoform is, in fact $S1P_1$ (Matloubian et al., 2004). Interestingly, mice lacking the *S1p1* gene specifically in hematopoetic lineages show defects in lymphocyte trafficking very similar to those seen in FTY720 treated mice. This result was unexpected as FTY720 is an S1P receptor agonist and therefore would be expected to result in a phenyotype opposite of *S1p1* knockout.

Why then does treatment with an agonist of S1P receptors result in a phenotype similar to loss of receptor function? The answer to this question relates to the events that occur after GPCR signaling is activated in a cell. One of the first thing that happens to many activated GPCRs is that they are removed from the cell surface and de-sensitized to their ligand (reviewed in von Zastrow, 2001). When internalization occurs the receptor can be targeted to several pathways, ranging from degradation to recycling to the plasma membrane. However, recycling to the membrane is unlikely to occur if the receptor is constantly activated by the presence of ligand or agonist. Therefore, in response to chronic FTY720-P exposure, $S1P_1$ is internalized and is unable to further interact with its



ligand (Graler and Goetzl, 2004). As a result, a compound that activates $S1P_1$ signaling, in fact, can act as a negative regulator of that signaling in vivo, by targeting the receptor for internalization and potentially degradation.

The reason downregulation of $S1P_1$ signaling results in sequestration of T and B cells to secondary lymphoid organs is also an interesting question. The answer to this question has to do with the natural expression cycles of $S1P_1$. It turns out that lymphocyte expression of $S1P_1$ cycles with the state of the cell (Lo et al., 2005; Matloubian et al., 2004). Circulating T cells express high levels of $S1P_1$. However, when activated, T-cell down regulate $S1P_1$ expression and home to the secondary lymphoid organs. Prior to egress from the lymph node, T cells once again upregulate $S1P_1$ receptor expression. Gain of function studies have further shown that T cell egress from the lymph node is accelerated when those T cells overexpress $S1P_1$ (Lo et al., 2005). In the case of B cells, $S1P_1$ signaling is involved in maintaining the appropriate distribution of this cell type in the marginal zone of the spleen. Again, in the case of FTY720 treated animals, B cells are mislocalized in secondary lymphoid organs. In the case of B cells however, the cells are unable to maintain themselves in the marginal zone of the spleen and move inappropriately to the splenic white pulp prior to activation (Cinamon et al., 2004).

One model for the ability of S1P to act in T cell egress from lymph nodes would be that the lipid acts as a chemoattractant for T cells expressing $S1P_1$. This hypothesis certainly fits with the $S1P_1$ mediated chemotactic responses discussed earlier in this chapter. For this to be the case, however, there must be an S1P concentration differential between the lymphoid organ and the blood stream. In fact, this is the case. Low S1P

levels are maintained in the lymph node by the degradative actions of S1P lyase (Schwab et al., 2005). Pharmaceutical disruption of the activity of S1P lyase, much like FTY720 treatment, results in lymphopenia and T cell sequestration in the lymph nodes. Presumably, this sequestration is due to the inability of T cells to sense the gradient of S1P that should exist between lymph node and blood. The finding that S1P lyase is required for normal T cell egress from lymphoid organs presents another exciting possibility for immunosuppressant therapies. Furthermore, because the mechanism by which S1P lyase inhibition affects T cell localization is slightly different than that of FTY720, these two approaches may be useful in a complementary fashion.

1.VIIIb Sphingosine and lymphocyte maturation

In addition to the important role S1P and its receptor, $S1P_1$, play in lymphocyte trafficking, these molecules have an earlier role in maturation of T cells. T cell precursors, upon leaving the bone marrow, take up residence in the thymus to continue with their further development. These thymocytes must undergo a series of differentiation steps before becoming mature $CD4^+$ or $CD8^+$ T cells. As was stated before, animals lacking functional *S1p1* gene in lymphocytes have a marked decrease in the number of circulating mature T cells. However, this decrease in the number of circulating T cells is not entirely due to the sequestration of these cell types in the secondary lymphoid organs, but also is partially due to the failure in maturing thymocytes to leave the thymus (Allende et al., 2004; Matloubian et al., 2004). Examination of the dynamics of $S1P_1$ receptor expression in thymocytes shows that the receptor first appears

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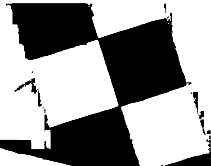
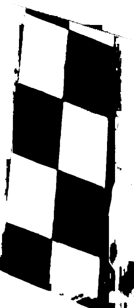
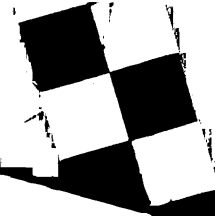
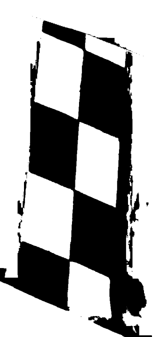



on $CD4^+$ $CD8^+$ thymocytes. However, it is not until the stage at which the T cell has chosen between $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ fates that $S1P_1$ expression peaks (Matloubian et al., 2004). Loss of $S1P_1$ expression results in the accumulation of mature T cells in the thymic medulla. Though the sequestered *S1p1*$^{-/-}$ thymocytes display markers of differentiated T cells, they fail to down regulate some markers of immature thymocytes (Alfonso et al., 2005; Matloubian et al., 2004). These findings suggest that, in addition to its putative chemotactic role, $S1P_1$ also plays an instructive role in the differentiation of thymocytes.

1.IX. Perspectives

Recently the number of research papers devoted to the topic of S1P signaling has expanded greatly. In part this is due to the discovery of the biomedical relevance of S1P and the identification of pharmacological agents that allow the manipulation of signaling. The identification of synthetic agonists and antagonists has made many of the recently published studies possible. In addition, the number of genetic tools available to study S1P receptors has increased. It will be interesting to see the directions into which research into S1P signaling will evolve. In addition to the important roles S1P receptor agonists are likely to play in organ transplantation, there are untapped potentials for S1P mediated signaling in cardiovascular medicine.

In addition to the processes I have covered in this chapter, it should be pointed out that S1P may have critical roles in the formation and function of the nervous system (Ishii et al., 2004). Furthermore, $S1P_1$ and $S1P_2$ have been linked to mast cell activation

and the allergic response (Jolly et al., 2004). The role S1P receptors play in the pathology of the allergic response and its derivative diseases, such as asthma, may prove to be critical areas of investigation for the medical communities dedicated to these pathologies.

Certainly the future will hold exciting new discoveries into S1P signaling. Not only will we identify new biological processes controlled by this fascinating lipid, we will also identify the unique partners that make this signaling possible. These discoveries will strengthen both clinical and basic sciences.

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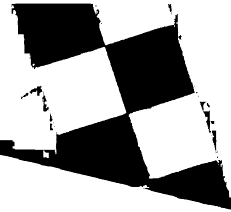
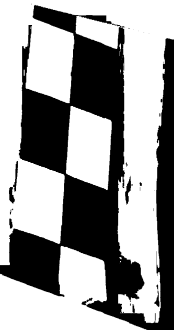



CHAPTER 2: Defects in the anterior endoderm of *miles apart* affect heart and jaw development

2.I. Introduction

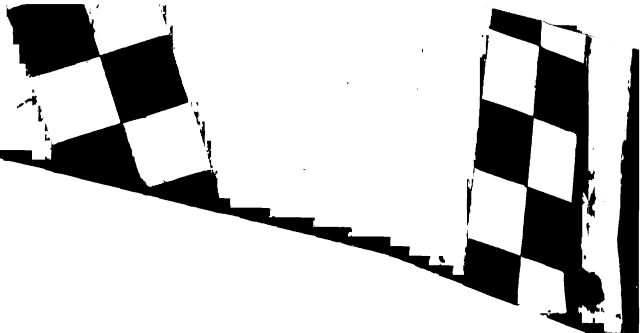
In vertebrate embryos, the heart is one of the first organs that must function for embryonic viability. The assembly of the heart is a complicated process that involves interplay between the cellular signaling that specifies the cell types of the heart, differentiation factors involved in translating specification signals into the appropriate cardiac gene expression patterns and, finally, complex cell movements that generate the functioning organ from the specified cells (Stainier, 2001). While I will not discuss the specification and differentiation of the cells giving rise to the heart, it is interesting to note that downstream of specification of precardiac mesoderm (PCM), the processes of differentiation and morphogenesis occur largely independently of one another. Studies in the zebrafish have been critical in discovering the molecular determinants controlling both differentiation and cardiac morphogenesis.

One area in which the zebrafish has shown exceptional utility is in the study of the migration of the PCM, one of the events required for early cardiac morphogenesis. In zebrafish, as in all vertebrates, the cells that give rise to the myocardial layer of the heart arise as bilateral populations in the anterior mesoderm (Figure 2.1A). During the mid-somitogenesis stages of development (approximately 15-19 hpf), these bilateral populations migrate medially. Upon reaching the midline, the fields of myocardial precursor cells fuse to form the primitive heart tube (19 hpf). During the process of migration the myocardial precursors not only undergo medial migration but also interact

with one another to form an epithelial sheet. Formation of this epithelial sheet is required for proper migration of the PCM (Trinh and Stainier, 2004). Although the process of PCM migration is quite complex the zebrafish has several characteristics making it an excellent system for studying this process.

Zebrafish have unique advantages for such study, both in the simplicity of the embryonic tissue architecture and in the powerful genetics offered by this system. Although other tissues may signal to the PCM during migration, there are only four tissues that are likely to play a physical role in this process (Figure 2.1B). First, there are the cells of the PCM itself, the myocardial (red) and endocardial (green) precursor cells. These cells are both derivatives of anterior lateral mesoderm that arise as bilateral populations and migrate to the midline to form the heart. In addition to these cell types, there is also the anterior endoderm. The anterior endoderm (blue) is the embryonic tissue in direct contact with the PCM as it migrates. Anterior endoderm is necessary for the migration of the PCM (Alexander et al., 1999; Kikuchi et al., 2000; Reiter et al., 1999) and may function as a signaling center as well as physical scaffold for this migration. In addition to embryonic tissues, the yolk syncytial layer (YSL), an extraembryonic syncytium, is in contact ventrally with the migrating PCM. Given its close association with the PCM, the YSL may also play a physical role as a substrate for PCM migration.

The second powerful advantage to zebrafish is the use of forward genetics to uncover novel genes involved in PCM migration. Currently, there are 9 mutants known to affect this process: *one-eyed pinhead (oep)/egf-cfc, casanova (cas)/sox32, bonnie and clyde (bon)/mix-type homeodomain gene, faust (fau)/gata5, hands-off (han)/hand2, natter (nat)/fibronectin, two of hearts (toh),* and *miles apart (mil)/s1p2* (Stainier, 2001). These



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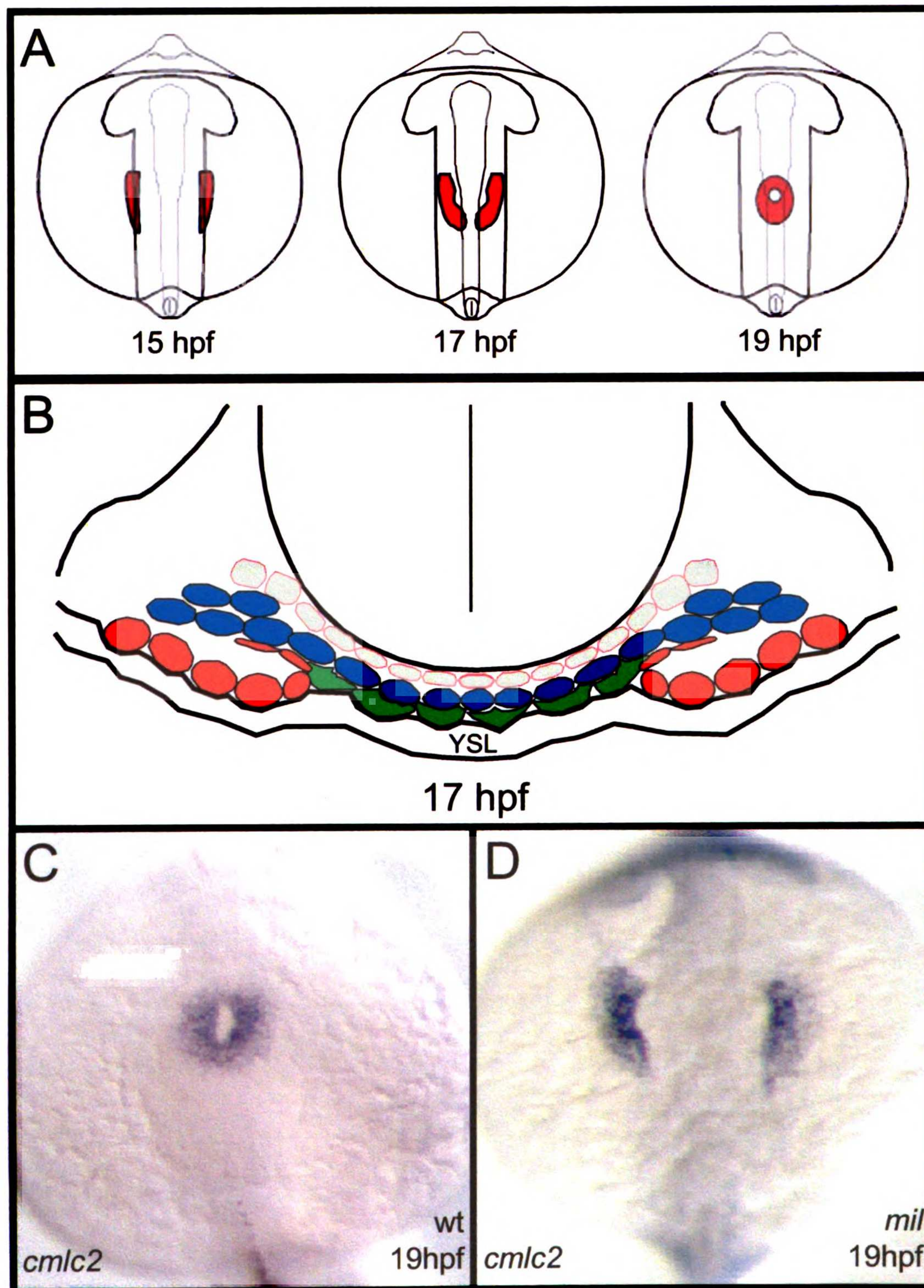

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Figure 2.1: Overview of precardiac mesoderm migration in zebrafish

(A) Schematic representations of zebrafish embryos at the indicated stages of development. Red represents the myocardial cells as they migrate from lateral positions to the embryonic midline. Dorsal views with anterior up. (B) Schematic representation of a crossection through the heart forming region of zebrafish embryos at 17 hpf. Red represents myocardial cells. Green represents endocardial cells. Blue represents anterior endoderm. Gray cells dorsal to the endoderm are head mesenchyme cells. (C and D) Visualization of the myocardial precursor cell populations in embryos 19 hpf by expression of *cmlc2*. In wild-type embryos (C) the myocardial precursors form the ring-like primitive heart tube. In *mil* mutant embryos (D) the myocardial precursors have failed to migrate and remain as bilater populations. Dorsal views with anterior up.

mutants can be broken into several categories. *oep, cas, bon* and *fau* all effect the specification and differentiation of the endoderm. *fau* can also be placed with *han* in a group of mutations affecting the differentiation of myocardial cells. The *nat* mutant disrupts PCM migration by causing defects in the formation of polarized epithelium by the PCM. Finally, *toh*, which will be discussed in Chapter 3 of this dissertation, and *mil* form a group of mutants in which there is no evidence that specification or differentiation of either the endoderm or PCM are affected.

The zebrafish mutant *mil* gave the first genetic evidence for the involvement of an S1P receptor in the development of the vertebrate cardiovascular system (Kupperman et al., 2000). The *mil* locus, as was previously noted, encodes a zebrafish orthologue of the mammalian $S1P_2$ receptor. At 19 hpf the myocardial ring of the primitive heart tube of wild-type embryos can be seen by expression of *cardiac myosin light chain 2 (cmlc2)* (Figure 2.1C). In *mil* mutant embryos at 19hpf the cells that would normally give rise to the primitive heart tube remain in lateral positions (Figure 2.1D). Cell transplantation studies using *mil* mutant embryos indicated that the *mil* gene was acting cell non-autonomously in the myocardial precursor cells during migration (Kupperman et al., 2000), suggesting that *mil* might act in one of the other cell and tissue types involved in this process. However, these experiments involved the transplantation of individual cells into the myocardial precursor cell populations and studying the behavior of the transplanted cells in host embryos. Because the myocardial precursor cells do not migrate as individuals, but as epithelial sheets (Trinh and Stainier, 2004), individual cells might be unable to escape the epithelium to demonstrate cell-autonomous behaviors. Therefore the tissue specific role of *mil* signaling remained somewhat unclear.

Because exact mechanism contributing to the PCM migration defects in *mil* mutants had not been resolved, I decided to investigate in detail how *mil* affects PCM migration. In this chapter I show that: 1) *mil* is specifically expressed in the anterior endoderm during PCM migration, 2) *mil* mutant embryos have defects in the morphogenesis of the anterior endoderm, 3) *mil* mutations affect the migration of the endocardial precursor cells in addition to myocardial precursor cells and 4) *mil* mutant embryos have defects in jaw development consistent with defects in anterior endoderm.

2.II. *miles apart* is expressed specifically in the anterior endoderm

Although transplantation studies have suggested that the *mil* gene functions cell non-autonomously in PCM migration, there was no evidence to suggest exactly where this gene was functioning. Wholemount in situ hybridization against the *mil* gene product (Figure 2.2A) narrows the likely tissues to a deep midline tissue or tissues in the anterior of the embryo and to the midbrain-hindbrain boundary (asterisk) (Kupperman et al., 2000). However, by wholemount visualization it is unclear if *mil* is expressed in the anterior endoderm, the YSL, or even in a combination of tissues including the PCM. In order to determine exactly what tissue(s) at the anterior midline of the embryo expresses *mil*, I sectioned zebrafish embryos stained for *mil* expression.

In crossection one can see that a thin strip of cells at the midline of the embryo expresses *mil* (Figure 2.2B). The cells expressing *mil* are the cells of the anterior endoderm (ae; see schematic in Figure 2.1B for comparison). Interestingly, the expression of *mil* is specific to these cells and is excluded from other cell types present in that region of the embryo, including myocardial cell precursors (mc; red dashed line),

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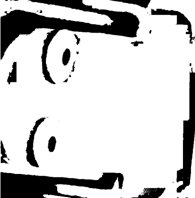

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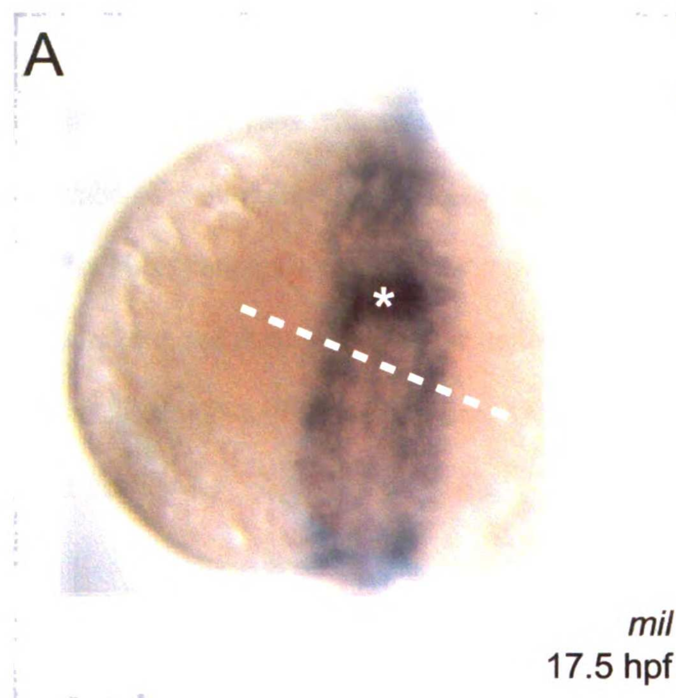

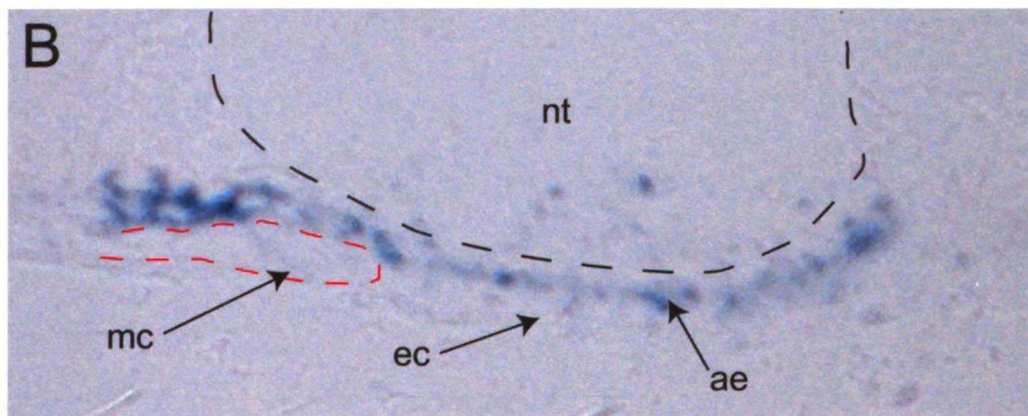

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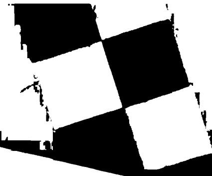
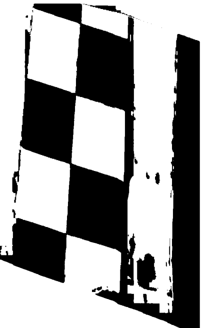
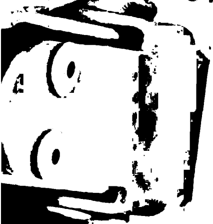
Figure 2.2: Expression of the *miles apart* gene in wild-type embryos

(A) Wholemount in situ hybridization against the *mil* gene. The asterisk indicates the midbrain-hindbrain boundary and the dashed line indicates the approximate position of the crossection in panel (B). Dorsal view with anterior up.

(B) Crossection of an embryos stained for expression of the *mil* gene at the level indicated in panel (A). *mil* is expressed in the anterior endoderm (ae) and is not in myocardial cell precursors (mc; red dashed line) or the endocardial cell precursors (ec). The neural tube (nt) is outlined with a black dashed line. The position of the yolk cell is indicated with the word Yolk.

endocardial cell precurors (ec), the YSL and the head mesenchymal cells immediately ventral to the neural tube.

These data support the previous claim that *mil* was unlikely to be acting in the PCM. Because *mil* encodes a G protein-coupled receptor (GPCR), the function of this gene would be predicted to be autonomous to the tissue in which it is expressed. Therefore, *mil* is likely to be acting in the endoderm. In previous analyses it was shown that the endoderm of *mil* mutant embryos was present and found in relatively normal positions within the embryo (Kupperman et al., 2000). It was noted, however, that in some severely affected *mil* mutant embryos the endoderm, while present, did show some morphogenetic defects at 24 hours post fertilization (hpf) (Kupperman et al., 2000). I therefore decided to investigate the morphogenesis of the anterior endoderm of *mil* mutant embryos during the stages of development when PCM migration is occuring.

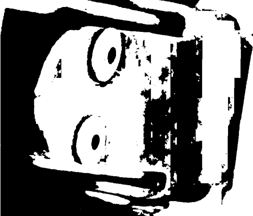
2.III. Anterior endoderm morphogenesis is defective in *miles apart* mutant embryos

In order to visualize the anterior endoderm of zebrafish embryos I utilized a transgenic zebrafish line expressing green fluorescent protein (GFP) under the control of a portion of the *her5* gene promoter. This promoter fragment had previously been shown to direct transgene expression specifically to the anterior endoderm (Tallafuss and Bally-Cuif, 2003). In wholemount visualization of these embryos one can clearly see the morphology of the anterior endoderm of both wild-type (Figure 2.3A) and *mil* mutant embryos (Figure 2.3B) at 17 hpf.

In wild-type embryos, the anterior endoderm forms a contiguous sheet of cells that span the entire embryonic midline. In *mil* mutant embryos, in contrast, there were

several defects that had not been previously noted. The most critical difference between wild-type and *mil* mutant embryos is that *mil* mutant embryos have gaps (arrowheads) in their anterior endodermal sheet. In addition to developing gaps, the anterior endoderm of *mil* mutants spreads farther laterally than wild-type embryos (bar). The defects in *mil* mutant endoderm morphogenesis are so severe that the most anterior aspect of the embryo is usually devoid of any *her5::gfp* positive endodermal cells (asterisk). Therefore, although initial specification and differentiation of the endoderm is normal in *mil* mutant embryos, morphology of the anterior endoderm is defective during the stages of PCM migration.

To examine the exact physical relationship between myocardial precursors and the endoderm in *mil* mutants, I sectioned both wild-type and *mil* mutant *her5::gfp* transgenic embryos at 19 hpf and counterstained these embryos for β-catenin (Figure 2.3C and D, respectively). As seen in wholemount visualization, wild-type endoderm forms a sheet of continuous cells across the embryonic midline (Figure 2.3C). Furthermore, one can see that these endodermal cells are rather thin in crossection as they spread out and contact one another. In contrast, *mil* mutant embryos show frequent gaps in their anterior endodermal sheet (Figure 2.3D, arrowhead). These gaps in endoderm generally coincide with the medial most migration of the myocardial precursor cells (dashed line), identified by their basolateral β-catenin localization (arrows). In addition to failing to extend across these gaps, the endodermal cells of *mil* mutant embryos often appear more rounded in shape and less compressed dorso-ventrally, as compared to wild-type endodermal cells. Therefore, there are clear differences in the anterior endodermal morphology, both at the gross as well as the cellular level, between wild-type and *mil*



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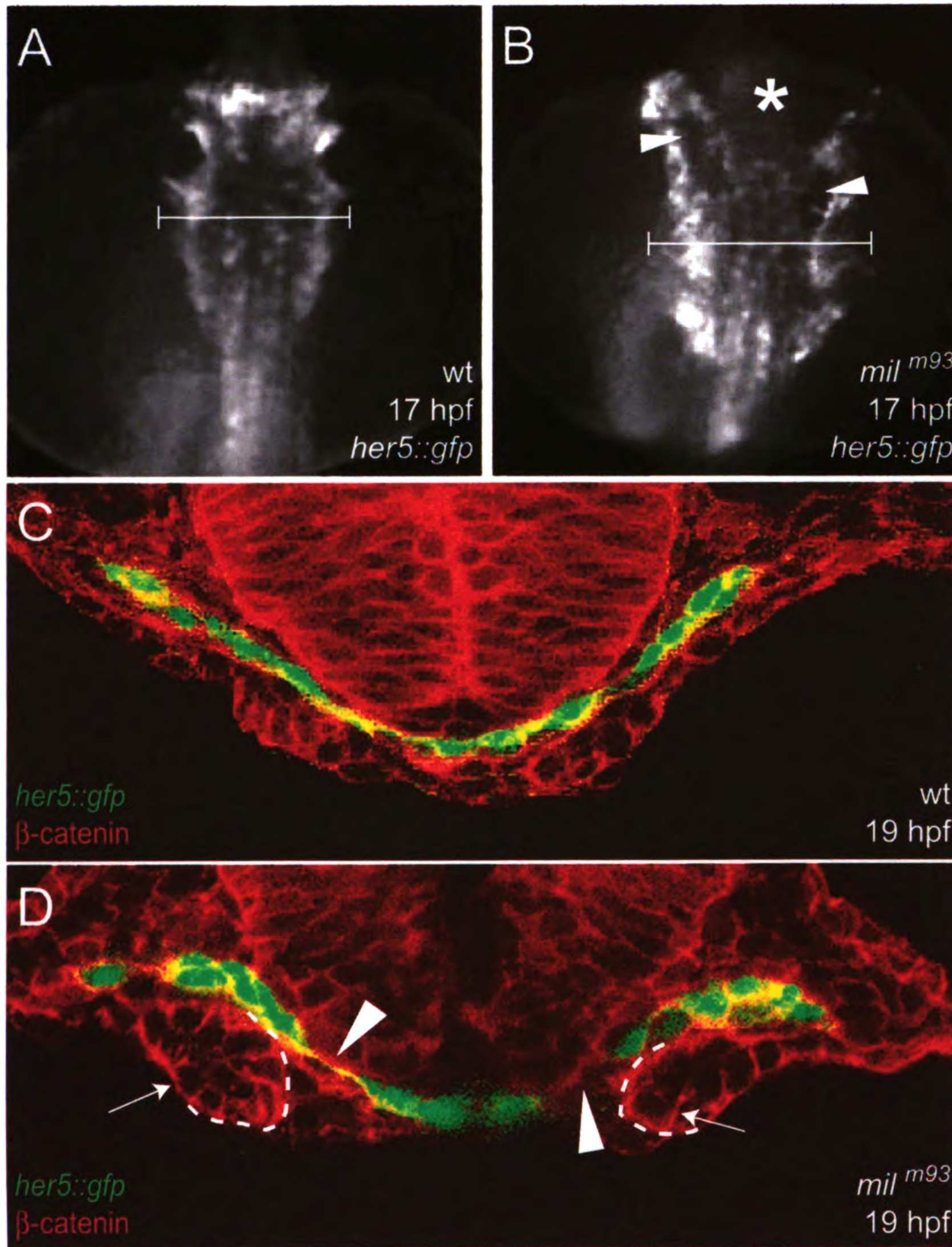

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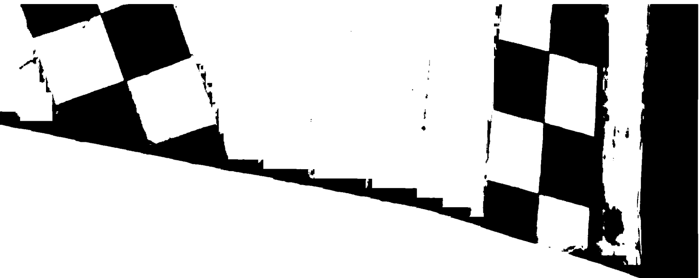

Figure 2.3: Anterior endoderm is disrupted in *miles apart* mutant embryos

(A and B) Visualization of anterior endoderm by expression of *her5::gfp* transgene. In wild-type embryos (A) the endoderm is continuous across the midline and has a narrower lateral extent (bar). In *mil* mutants (B) the endoderm has gaps with no cells expressing GFP (arrowheads) and the lateral extent of the endoderm is wider than wild-type embryos (bar). Dorsal view with anterior up. (C and D) Confocal analysis of crossections of embryos at 19 hpf expressing *her5::gfp* in endodermal cells (green) and counterstained with anti-β-catenin antibody (red). Wild-type embryos (C) have a continuous endoderm spanning the mid-line. In addition myocardial cells form a polarized epithelium, indicated by basolateral localization of β-catenin staining (arrow). In *mil* mutant embryos (D) the endoderm is discontinuous across the midline. Gaps (arrowhead) can be found that predict the medial-most migration of precardiac mesoderm. Myocardial cells have normal epithelial polarity, indicated by basolateral β-catenin localization (arrows).

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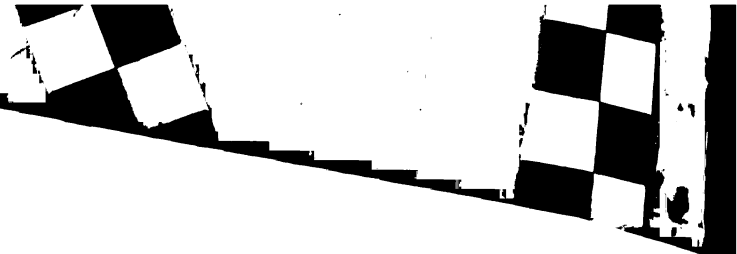

mutant embryos. I suggest that the defects in heart formation of ***mil*** mutant embryos result from primary defects in anterior endodermal morphogenesis.

Myocardial precursor cells, however, are not the only cells in the embryo that require the endoderm to migrate properly. For instance the endocardial cell precursor populations are also closely associated with the anterior endoderm (Trinh and Stainier, 2004). In addition, later in development, the craniofacial cartilaginous elements of the zebrafish require patterning signals and support from the anterior endoderm (David et al., 2002a). Therefore, if the endoderm is severely affected in *mil,* then endocardial cells and craniofacial development would also be expected to have defects. Therefore, to verify that the anterior endoderm was significantly disrupted in *mil* embryos, I examined both endocardial cell precursor morphogenesis and craniofacial development.

2.IV. Endocardial cell migration is defective in *mil* mutant embryos

To examine the endocardial precursor cell populations of zebrafish embryos, I used the *flk1::gfp* transgenic line (Field et al., 2003). Wholemount visualization of *flk1::gfp* transgenic embryos at 17 hpf shows clear differences between uninjected (Figure 2.4A) and ***mil*** morpholino injected embryos (morphants; Figure 2.4B). At 17 hpf, the endocardial cell (ec) precursor population has migrated to the midline of uninjected embryos. These precursors form a cluster of GFP positive cells at the embryonic midline and will eventually be surrounded by the ring-shaped myocardium of the primitive heart tube. In ***mil*** morphants, at the same stage of development, no such cluster of endocardial precursor cells can be found at the embryonic midline. However, on either side of the embryo there are bright clusters of *flk1::gfp* positive cells that are most likely the

endocardial cell precursors (ec?). Although I could not verify, by wholemount examination, that these are, in fact, endocardial precursor populations.

Because it was difficult to verify which cells corresponded to the endocardial precursor cells, I sectioned *flk1::gfp* transgenic embryos through the PCM at 17 hpf to examine the cellular relationships between the endocardial precursor cells and their surrounding tissues (Figure 2.4C and D). Under higher resolution confocal analysis, one sees that, the *flk1::gfp* transgene is expressed in the lateral anterior endoderm (lae) in addition to the endocardial precursor cell population (ec). These endodermal cells can be identified by morphology and the heightened cortical localization of β-catenin (Trinh and Stainier, 2004). In contrast, the more mesenchymal endocardial cell precursors have lower levels of cortical localization of β-catenin (Trinh and Stainier, 2004).

In uninjected embryos (Figure 2.4C), the endocardial precursors can be seen at the embryonic midline, medial to the migrating myocardial precursor epithelia. In contrast to uninjected siblings, the endocardial cell precursors of *mil* morpholino injected embryos (Figure 2.4D) have not migrated medially and are found in more lateral regions of the embryo. In *mil* morphants endocardial precursor cells are found in contact with two cell types. The most medial endocardial precursors are found in contact with endodermal cells. However, another population of endocardial precursor cells is found more laterally and is in contact with myocardial precursor epithelia.

These data clearly show that, in addition to defects in the migration of the myocardial cell precursors, embryos lacking *mil* mediated signaling also have defects in the migration of endocardial precursor cells. Furthermore, these findings give support to

Figure 2.4

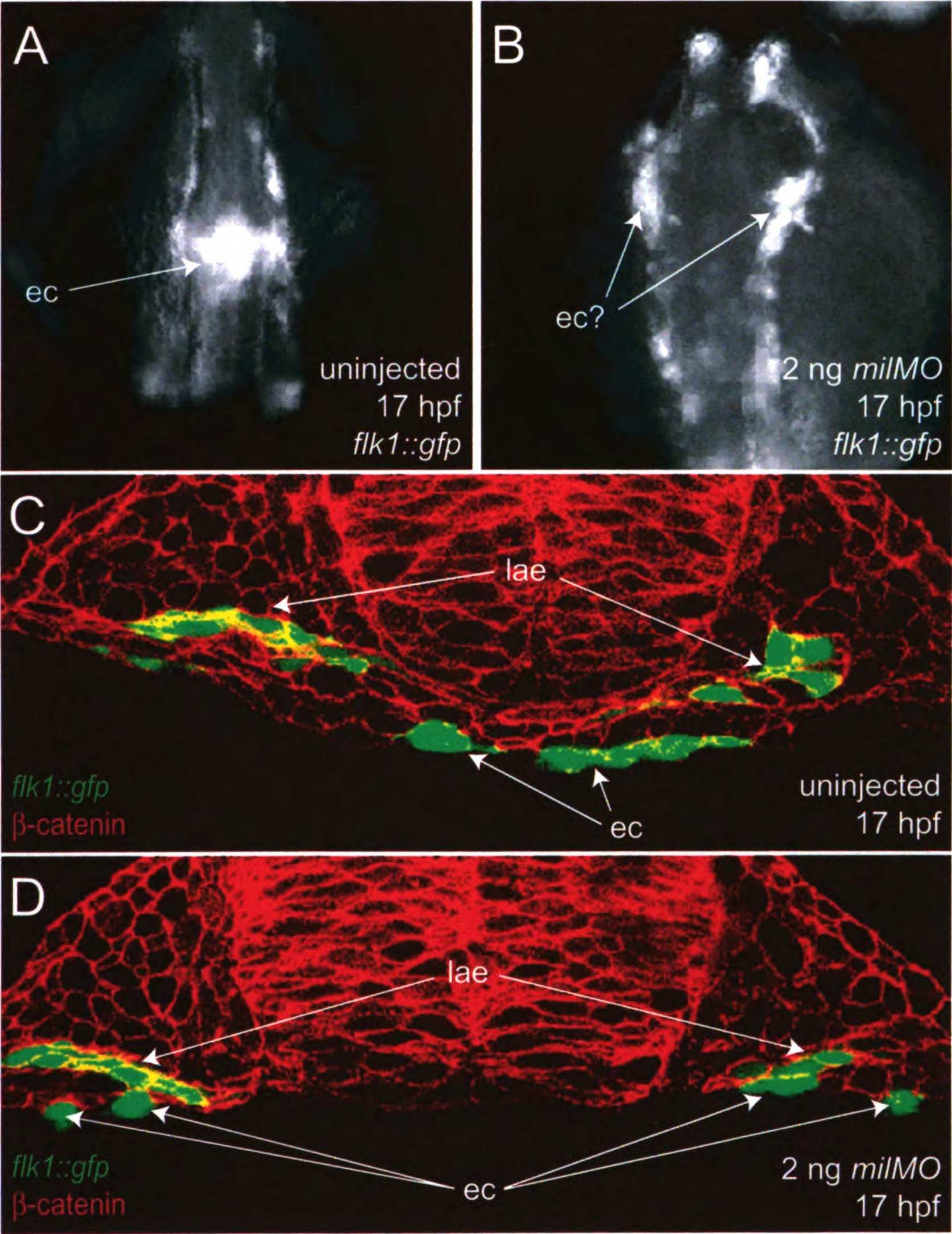

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Figure 2.4: Endocardial precursor migration is defective in *miles apart* morphants

(A and B) Wholemount visualization of *flk1::gfp* transgene expression at 17 hpf. In wild-type embryos (A) the endocardial cell precursor population (ec) has migrated to the midline and form a cluster of cells. In *mil* morpholino injected embryos (B) ec populations fail to migrate medially. Dorsal views with anterior up. (C and D) Confocal analysis of crossections through *flk1::gfp* transgenic zebrafish. By confocal analysis the *flk1::gfp* transgene (green) marks both endocardial cell precursors (ec) and a portion of the lateral anterior endoderm (lae). lae cells are identified by heightened cortical localization of β-catenin staining (red). ec precursors have little or no cortical β-catenin localization.

the hypothesis that the anterior endoderm defect is the primary defect and later results in defects in heart formation in embryos with defective *mil* gene function.

2.V. *miles apart* mutant embryos have defects in craniofacial development

In addition to the defects seen in the migration of the myocardial and endocardial cell precursors, I examined whether or not the *mil* mutation affects a process later in development that is also dependent on the endoderm, namely, the development of the craniofacial cartilaginous elements (David et al., 2002a). Wild-type embryos stained with alcian blue (Figure 2.5A and B) display the normal pattern of jaw and gill arch elements, as well as the neurocranium. However, *mil* mutant embryos stained at the same point in development (Figure 2.5C and D) show abnormal craniofacial development. The most interesting observation in *mil* mutant embryos is the complete loss of Meckel's cartilage (m), the jaw element that will eventually form the mandible. In addition to loss of Meckel's cartilage, the palatoquadrate (pq) of *mil* mutants is severely reduced in size. The palatoquadrate in *mil* mutants also has a truncated or absent palatoquadrate process (pp), suggesting that morphogenesis as well as size of this jaw element may be affected. Both Meckel's cartilage and the palatoquadrate are elements derived from the first pharyngeal arch (Schilling et al., 1996). It is interesting in that the elements of the jaw most severely affected in the *mil* mutant embryo are also derived from the most anterior pharyngeal arch. This coincides with the area of the anterior endoderm most affected in *mil* mutant embryos (see Figure 2.3B) and suggests a link between the defects in the endoderm and jaw in these mutants.

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Most of the more posterior pharyngeal arch derived elements appear to be present in *mil* mutant embryos. However, number of ceratobranchial (cb) elements may be reduced in *mil* mutants by one or two pairs. It is difficult to say for certain as alcian blue staining in *mil* mutants is often faint in certain elements making it difficult to see some of the posterior cartilaginous structures. For instance, the ceratobranchial and basibranchial (bb) elements in *mil* mutants are often weakly stained and difficult to visualize. In addition to the loss of elements, the entire craniofacial region of *mil* mutants are compressed anteriorly when looking at the distance between the ethmoid plate and the auditory capsule (ac; the posterior element of the neurocranium). The cause of this phenotype, however, may simply be the result of distorting pressure from the edema around the heart and surrounding tissues in *mil* mutant embryos at this stage of development, rather than a specific result of the loss of *mil* mediated signaling. Another possibility, however, is that there is a general compression of the head structures in *mil* mutants due to a lack of circulation. Other cardiovascular mutants, such as the *cardiac troponin T* mutant *silent heart (sih)*, have small heads. However, in *sih* mutants all of the craniofacial elements are present and appear normally patterned (not shown).

In addition to the defect in the elements of the jaws and gill arches of *mil* mutant embryos there also affects the ethmoid plate (e), the anterior most projection of the neurocranium. In wild-type embryos, the ethmoid plate appears as a single, fused element dorsal to Meckel's cartilage. In *mil* mutants, however, the ethmoid plate fails to fuse and is made up of two elements. This defect is reminiscent of defects in mutants known to disrupt formation of the zebrafish midline, such as *sonic hedgehog (shh)* (Brand et al., 1996). However, examination of the migration of neural crest cells by *dlx2*

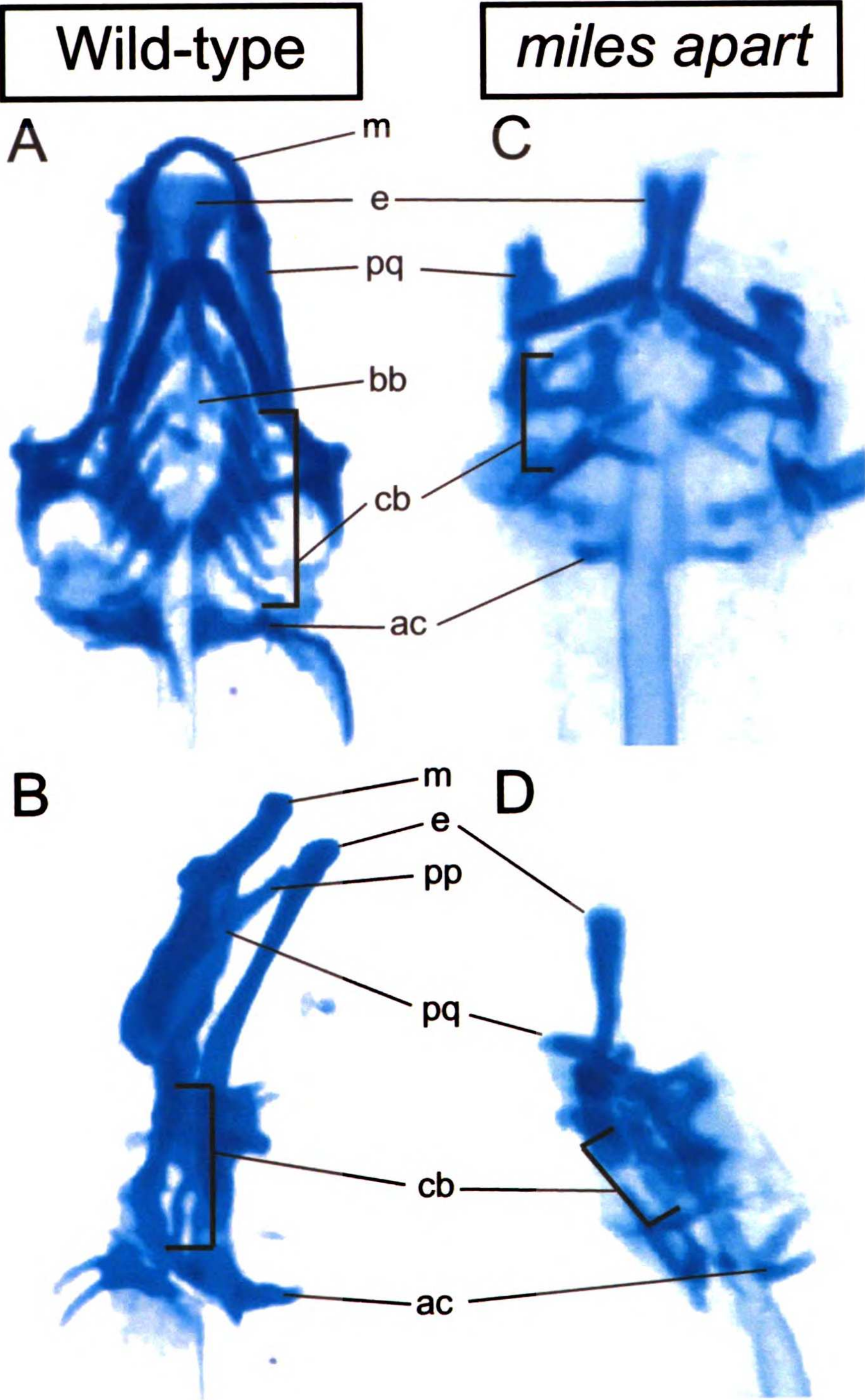
expression indicates that the neural crest populations that give rise to jaw elements are unaffected in *mil* mutant embryos (data not shown). This suggests that *mil* affects craniofacial elements in a different fashion than *shh*, as hedgehog signaling is thought to affect this process in great part by disrupting neural crest derivatives (Wada et al., 2005).

Two other zebrafish mutants have been shown to affect the development of the neurocranium and jaw elements in a fashion somewhat similar to *mil*, *cas* and *bon*. Both *cas* and *bon* mutations affect the specification of endoderm in zebrafish. In *cas* mutants the endoderm is completely absent (Alexander et al., 1999), while in *bon* mutants the endoderm is severely depleted but partially present (Kikuchi et al., 2000; Trinh et al., 2003). In *cas* mutants the elements of the jaw and gill arches are almost completely absent and the neurocranium is dysmorphic. In *bon* mutants the jaw elements appear affected to a similar extent as *cas* mutants, although the neurocranial defects are less severe (David et al., 2002a). *mil* mutants are even less severely affected than either *cas* or *bon* mutant embryos. These mutants all affect the anterior endoderm to different extents. Interestingly, the severity of the effects of each of these mutants on the endoderm mirrors the relative severity of their effects on craniofacial development, with *cas* having the most severe effects and *mil* having the least severe. Therefore, it is likely that the defects in *mil* mutant craniofacial development are the result of the defects seen in anterior endoderm development, as is the case with *cas* and *bon*.

2.VI. Perspectives and future directions

It is becoming increasingly clear that the defects seen in the formation of the heart in *mil* mutant zebrafish are likely to be secondary effects due to defects in the anterior

Figure 2.5



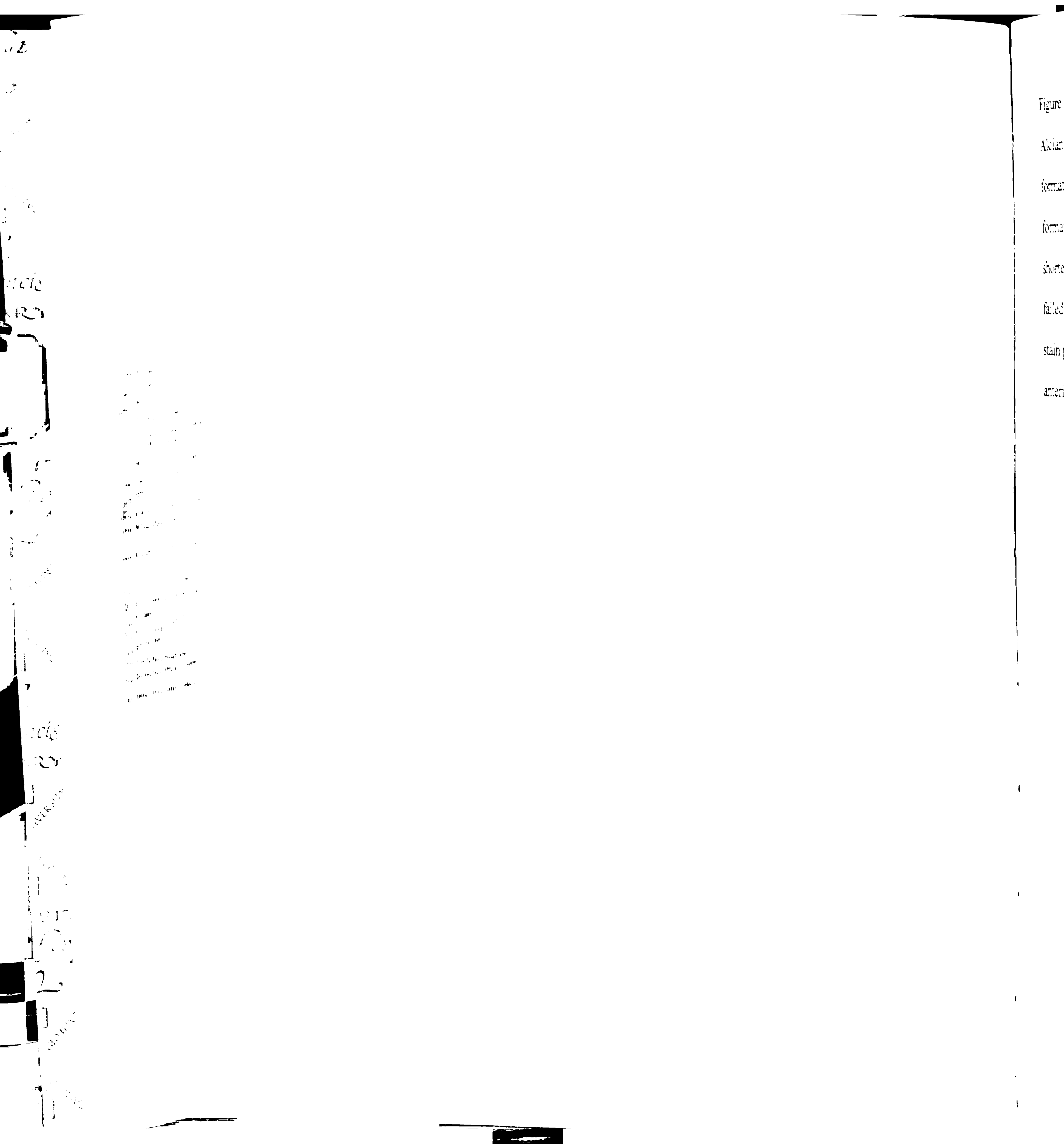


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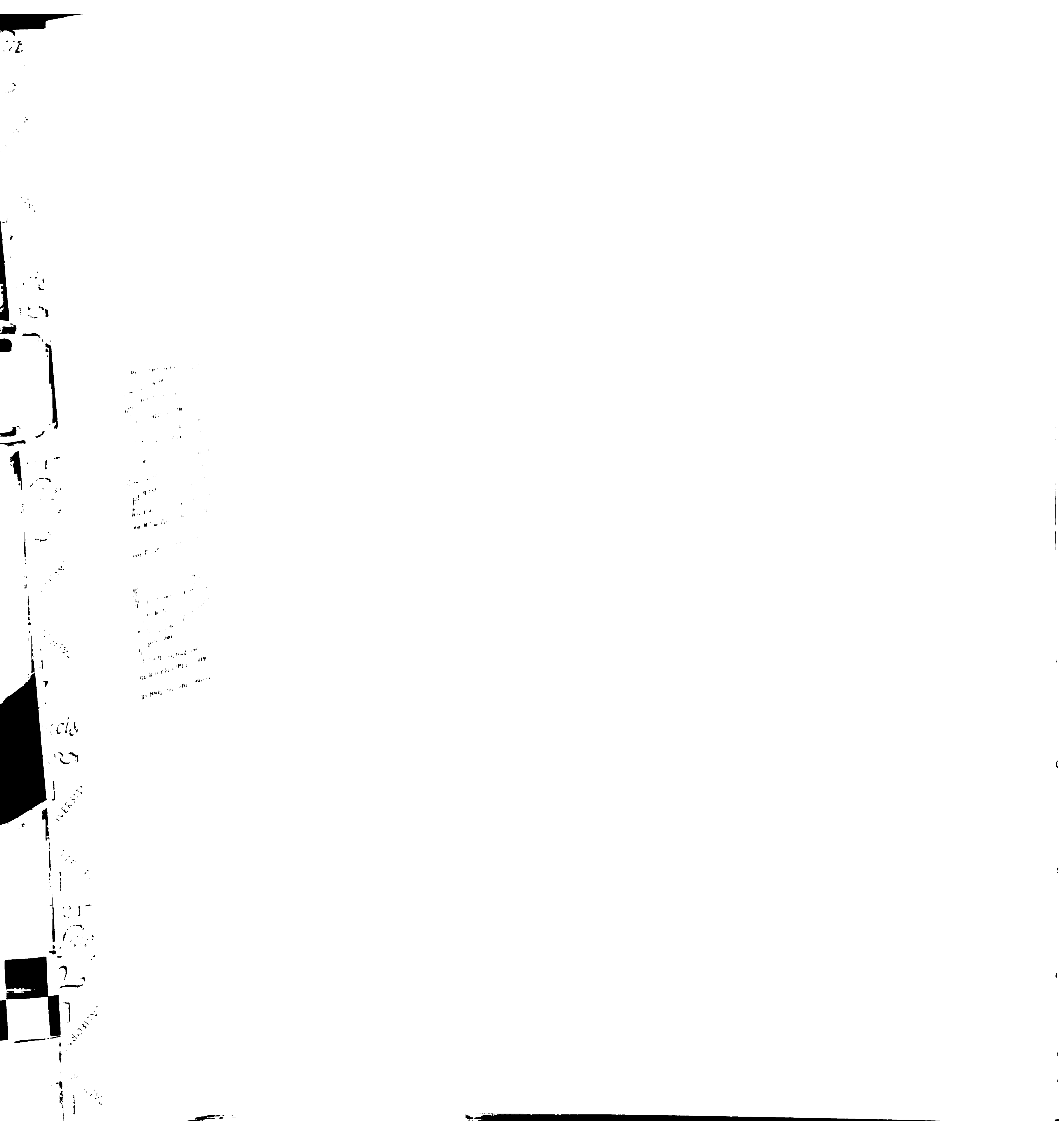
Figure 2.5: *miles apart* embryos display defects in jaw development

Alcian blue staining of wild-type embryos at 120 hpf (A and B) shows normal cartilage formation. At 120 hpf, *mil* mutant embryos (C and D) have defects in jaw cartilage formation. In *mil* mutants Meckel's cartilage (m) is absent, the palatoquadrate (pq) is shorted and is missing the palatoquadrate process (pp) and the ethmoid plate (e) has failed to fuse. While present, the ceratobranchial (cb) cartilages of *mil* mutant embryos stain poorly. (ac) auditory capsule; (bb) basibranchial. (A and C) Ventral views with anterior up. (B and D) Lateral views with anterior up.

endoderm. This hypothesis is supported by the fact that during the migration of PCM to the embryonic midline, the only tissues in the anterior of the embryo expressing *mil* are the anterior endoderm and the midbrain-hindbrain boundary. It is unlikely that the midbrain-hindbrain boundary is involved in the process of PCM migration because mutants affecting midbrain-hindbrain boundary establishment do not have PCM migration defects (Lun and Brand, 1998; Reifers et al., 1998). Therefore it is probable that *mil* mediated signaling is acting within anterior endodermal cells.

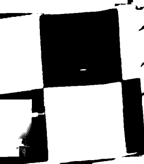
The hypothesis that *mil* functions in the anterior endoderm is further supported by the fact that there are clear morphogenetic defects in the anterior endoderm during the stages of development when PCM migration is occurring. Defects in morphogenesis of the anterior endoderm in *mil* mutants result in gaps in the endodermal sheet. Because the cells giving rise to the heart migrate in close apposition to the endoderm, it is probable that these gaps create boundaries to migration of PCM.

Finally, I present here that *mil* mutants also display defects in craniofacial development that would be predicted if *mil* mutants had primary defects in the anterior endoderm. Experiments with the endoderm mutants *cas* and *bon* gave direct evidence that the endoderm was required for zebrafish craniofacial development (David et al., 2002a). As was mentioned above, *cas* and *bon*, have similar effects to *mil* on the development of the neurocranium, but have much more severe effects on the pharyngeal arch derivatives of the jaw and gills. The more severe effects of *cas* and *bon* mutations on jaw and gill arch elements are probably due to the more severe effects these mutations have on endoderm. Therefore, *mil, cas* and *bon* mutants may represent a series of mutants that cause craniofacial defects of similar etiology, but differing severity.



Important questions remain with regards to the function of *mil* in the process of PCM migration. For instance, while it is clear that *mil* mediated signaling in the endoderm affects the morphology of that tissue, it is not known how *mil* affects endoderm morphogenesis on a cellular level. Given the nature of the *mil* gene product, some hypotheses can be made as to how *mil* functions. As *mil* encodes an orthologue of the mammalian $S1P_2$, we can make some predictions about the signaling partners of Mil, however.

One of the most studied roles of $S1P_2$ is the role this receptor plays in activation and inhibition of Rho family small GTPases (See Chapter 1.Vb). These GTPases are potent modulators of the cytoskeleton and cell adhesion (Evers et al., 2000). Therefore, it is possible that *mil* might affect endodermal morphology via the control of cytoskeletal organization or by regulation of cell adhesion molecules. One indication that the cytoskeleton of *mil* endodermal cells could be defective is shape of these cells in crossection. As was mentioned earlier wild-type endodermal cells are spread thinly across the midline. In contrast, *mil* endodermal cells appear more rounded in shape. The rounded morphology of *mil* endodermal cells could be due to a misregulation of the cytoskeleton. It is also possible that the cell shape defects in these cells are due to problems with cell-cell or cell-matrix adhesion. I have been unable to find any obvious defects in cell adhesion molecules in the endoderm of *mil,* however. For example, the endoderm of *mil* mutants has normal upregulation of cortical β-catenin localization, indicating an upregulation of cadherin mediated adhesion. This result is verified by staining with a pan-cadherin antibody (not shown), as well. Unfortunately, due to a lack

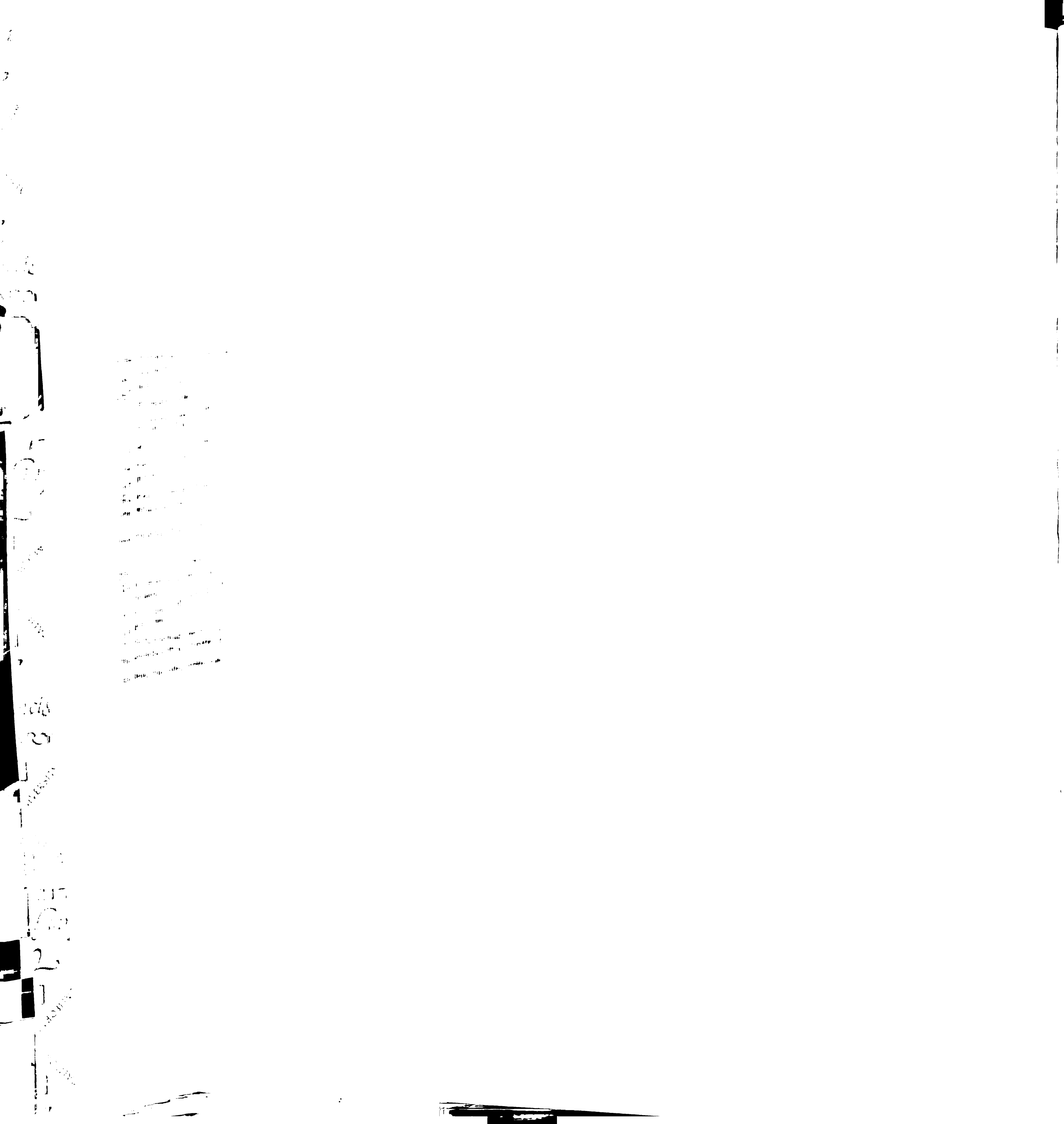


of reagents, I have been unable to investigate whether *mil* mutant endodermal cells have defects in the integrin class of cell adhesion molecules.

In chapter 1 of this dissertation I also discussed how the expression of $S1P_2$ was often refractory to cell migration. If Mil mediates signaling in a similar manner to $S1P_2$, as would be suggested by the predicted orthology of these receptors, then this anti-migratory signaling could constitute another mechanism by which Mil might affect anterior endoderm morphology. During gastrulation and early somitogenesis the endoderm is highly migratory, moving actively to the appropriate position in the embryo. Once the endoderm has reached the appropriate place, however, those migratory responses have to be turned off. If *mil* is required in the endoderm to attenuate migratory behavior, then perhaps loss of *mil* function leads to an extended and inappropriate migratory phase in the endoderm. The gaps that form in the endodermal sheet may simply be holes formed as endodermal cells move away from one another in response to chemotactic signals that would normally be antagonized by Mil.

Another question that I have not investigated in this study is the exact timing of the appearance of endodermal defects in *mil* mutant embryos. The expression of *mil* becomes restricted to the endodermal cell layer between 10 and 12 hpf. The change in expression of *mil* from general to specific might indicate the phase of development when *mil* function becomes necessary in the endoderm. Knowing the exact timing of the requirement for *mil* in the endoderm would be helpful as it would indicate when to look for cellular differences between wild-type and *mil* mutant embryos.

The zebrafish *mil* mutant has given a unique glimpse into questions of how tissues interact with one another during the complex movements required to form vertebrate



organs. This mutant also promises to teach us some of the mechanisms by which S1P can modulate cell behaviors in vivo. Through the use of the powerful genetics, embryology and imaging techniques available in zebrafish, I have shown that *mil* mutations affect the morphology of not only the heart, as was previously reported, but also of the anterior endoderm and even the elements of the jaw, gills and neurocranium later in development. What remains to be seen are the cellular mechanisms by which the zebrafish $S1P_2$ orthologue Mil affects the morphology of the endoderm. Finally, it also remains to be seen how the function of *mil* within the cells of the zebrafish endoderm will relate to the function of $S1P_2$ in mammalian cells and whether what is learned from *mil* might have an impact on our understanding of how this family of S1P receptors effects cells in general.

2.VII. Materials and Methods

Zebrafish Strains and Care

Adult and embryonic zebrafish were raised and cared for using standard laboratory procedures (Westerfield, 2000). We used the following zebrafish mutant and transgenic strains: *mil*^{m93} (Kupperman et al., 2000), *mil*^{te273} (Kupperman et al., 2000), *tg(0.7her5::gfp)*^{ne2067} (Tallafuss and Bally-Cuif, 2003) *and tg(flk1::gfp)*^{s843} (Beis et al., 2005).

Immunohistochemistry, Fluorescence microscopy and confocal analysis

Embryos were fixed at room temperature for 1 hour in 4% Paraformaldehyde in PBS or overnight at 4C in 2% Paraformaldehyde in PBS. Mouse monoclonal anti-β-catenin

antibody (15B8, Sigma-Aldrich) was used at 1:500 dilution. All staining was carried out in PBS/1% Dimethyl Sulfoxide/1% Bovine Serum Albumin/ 0.1% Triton X-100. Sectioned embryos were mounted in 4% Seakem GTG Low Melt Agarose in PBS and sectioned at 200 μm on a Leica VT1000 Vibratome. Images were aquired using a Zeiss LSM5 Pascal confocal microscope. Wholemount fluorescence microscopy was performed with a Zeiss SteREO Lumar.V12 microscope.

In situ hybridization and alcian staining

Wholemount in situ hybridization was carried out as described (Alexander et al., 1998) using the following probes: *cmlc2* (Yelon et al., 1999) *and mil* (Kupperman et al., 2000). Alcian blue staining was carried out as described (Schilling et al., 1996) at 5 dpf in embryos fixed for 1 hour in 4% Paraformaldehyde in PBS at room temperature. Embryos were photographed using a Zeiss Axiocam camera.

Morpholino anti-sense oligonucleotide

The *mil* morpholino was designed and generated by Gene Tools, LLC. The morpholino (5'- AGA CGG CAA GTA GTC ATT CAG AGG G-3') overlaps the start ATG of the *mil* message. Embryos were injected at the one-celled stage with 2 ng of *mil* morpholino.

CHAPTER 3: The spinster homologue, Two of Hearts, is required for sphingosine 1-phosphate signaling in zebrafish

3.I. Introduction

The *Drosophila spinster (spin)* gene, also called *benchwarmer*, is the founding member of the *spinster-like* gene family, which encode members of the major facilitator superfamily (MFS) of transmembrane transporters (Dermaut et al., 2005; Nakano et al., 2001). Despite having sequence homology to MFS proteins, *spinster-like* gene products have no known substrate or transporter activity. However, *Drosophila spin* mutant embryos display defects in intracellular trafficking of lipids and sugars (Dermaut et al., 2005; Nakano et al., 2001; Sweeney and Davis, 2002). These defects are reminiscent of human lipid sorting disorders, such as Tay-Sachs and Niemann-Pick syndromes (Sanyal and Ramaswami, 2002), making this gene of particular interest. The Spinster protein localizes intracellularly to the late endosomal or lysosomal compartments (Sweeney and Davis, 2002). These are the cellular compartments in which sugars and lipids seem to inappropriately accumulate in *spin* mutants (Dermaut et al., 2005). Therefore Spinster localizes to the compartment affected by mis-targeting of these sugars and lipids. In addition, and possibly as a result of those trafficking defects, *spin* mutant flies develop defects in cell death control in the central nervous system (Nakano et al., 2001) and in regulation of neuromuscular junction size (NMJs) (Sweeney and Davis, 2002).

One of the more interesting suggestions from *Drosophila spin* mutants is the possibility that this gene may function in TGFβ signaling (Sweeney and Davis, 2002). In *spin* mutant larvae the number of boutons found per NMJ is increased. However,

expression of a dominant negative TGFβ receptor suppresses this phenotype. Unfortunately, the epistatic relationship of TGFβ receptor to *spin* may occur because TGFβ signaling acts far upstream of *spin* in the expansion of NMJ size and not because *spin* has a direct role in TGFβ signaling. However, these data do suggest the intriguing possibility of a role for a relatively unstudied intracellular transporter family in mediating cellular signaling pathways.

Almost no data exists with respect to the function of vertebrate *spinster-like* genes. One study suggests that overexpression of the human *spinster-like 1* gene may results in caspase-independent cell death (Yanagisawa et al., 2003). Furthermore this study has suggested the possibility that *spinster-like 1* interacts with the cell death factors Bcl-2 and Bcl-xl via a putative BH3 motif. However, this BH3 motif is buried in a predicted transmembrane domain and it is unclear how it would be available for binding to Bcl-2 or Bcl-xl. The only other information regarding the function of vertebrate *spinster-like* genes comes from work on the zebrafish mutant *not really started (nrs)* (Young et al., 2002). The *nrs* locus encodes a zebrafish *spinster-like 1* orthologue. Mutations in *nrs* cause early embryonic lethality due to apparent necrotic death in the extra-embryonic yolk cell. However, the mechanism controlling this cell death is not clear.

Therefore, while it seems that *spinster-like* genes are important regulators of cellular processes, such as lipid trafficking, cell death and possibly cell-cell signaling, there is little understanding of the exact function of these genes. In this chapter I describe the isolation of another zebrafish *spinster-like* gene, *two of hearts (toh)*. Furthermore I show that this gene is critical for formation of the zebrafish heart via its interaction with a

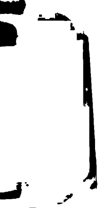
known signaling pathway. Finally, I show that the functions of this *spinster-like* gene are likely to have diverged from those of the *Drosophila spin* gene, suggesting that, in vertebrates, *spinster-like* genes may have evolved novel roles in specific signaling pathways that are important both developmentally and physiologically.

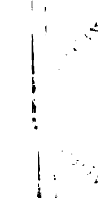
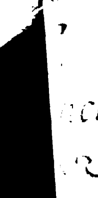
3.II. Isolation and Characterization of *two of hearts*

In zebrafish, as in all vertebrates, the primitive heart tube is formed from bilateral fields of precardiac cells located in the anterior lateral-plate mesoderm (Figure 2.1). The two cell populations migrate to the embryonic midline and fuse to form a single heart tube (reviewed in (Stainier, 2001). Although something is known about the genes involved in precardiac mesoderm (PCM) migration, this process is not entirely understood. In order to discover novel loci important for migration of the PCM, mutagenized lines of zebrafish were screened for mutations affecting the embryonic heart. Mutations in one such locus, *two of hearts (toh),* were found to lead to defects in the early development of the zebrafish heart. In this section, I discuss the nature of the heart defects in *toh* mutant embryos, the difference in phenotypic penetrance among the alleles of *toh* and the effect of maternal-zygotic *toh* mutations.

3.IIa. Characterization of the heart defects in *two of hearts* mutant embryos

At 36 hours post fertilization (hpf) wild-type zebrafish embryos have a well developed and functioning heart (Figure 3.1A). In contrast to wild type embryos, *toh* mutant embryos have defective circulation and, as a result, accumulate fluid in the space around their hearts, a condition called pericardial edema (Figure 3.1D, arrow). Circulatory



failure and consequential pericardial edema in *toh* mutant embryos is the result of a defect in migration of the PCM.

In wild-type zebrafish embryos at 19 hpf, the ring shaped primitive heart tube can be visualized using the myocardial cell specific marker *cardiac myosin light chain 2 (cmlc2)* (Figure 3.1B). At the same stage of development, the myocardial precursor cells of *toh* mutant embryos are delayed in their migration (Figure 1E). In some *toh* embryos the myocardial cell populations never fuse, resulting in the formation of bilateral heart-like structures, a condition called cardia bifida.

Although the myocardial precursor cells fail to migrate appropriately in *toh* mutants, the differentiation of these cells is unaffected. In fact, the heart structures in *toh* mutants display normal chamber specific gene expression and are infiltrated by the endocardial cells that would normally form the inner lining of the heart (not shown). However, these structures fail to form a normal lumen, have insufficient cardiac function to support circulation and do not connect appropriately to the vasculature. As a result, mutations in the *toh* locus are often lethal.

3.IIb. Penetrance of heart defects in *two of hearts* mutants is allele specific

The *toh* locus is represented by four mutant alleles, designated *s8, s220, s420* and *sk12*. The frequency of heart defects seen in *toh* mutants is dependent upon the allele in question. Overall, *s220* is the weakest allele, displaying only 22% penetrance of lethal heart phenotypes in heterozygous crosses (n = 5441 embryos). *s420* heterozygous carrier crosses, in contrast show 25% defective circulation (n = 1013 embryos), making this the most severely affected allele. The *s8* and *sk12* alleles show intermediate penetrance of

the heart phenotype (23%; n = 1597 and 23%; n = 1341 embryos, respectively). None of the alleles of *toh* shows full penetrance of true cardia bifida, as some embryos of each allele show partial fusion of the heart fields. However, the hearts resulting from partial fusion of the PCM are dysmorphic and fail to function properly. Because of the partial penetrance of the phenotypes seen, these *toh* alleles may represent hypomorphic mutations in the affected gene.

3.IIc. Maternal-zygotic *two of hearts* mutants have no additional phenotypes

Because *toh* mutant embryos of the *s220, sk12* and *s8* allele occasionally develop normal circulation, homozygous embryos can sometimes be raised to adulthood. These homozygous adults are fertile and mate normally. Incrosses of *s8* homozygous adult fish were used to generate maternal-zygotic *s8* ($MZtoh^{s8}$) mutant embryos. These embryos would be predicted to have a deficiency in maternally provided *toh* gene product as well as the gene product transcribed from the zygotic genome. $MZtoh^{s8}$ mutant embryos show no additional phenotypes when compared to zygotic *s8* mutant embryos. Because there are no additional phenotypes in $MZtoh^{s8}$ embryos, it is unlikely that *toh* function is required for processes earlier in development that are rescued by maternal contribution of the gene. However, all $MZtoh^{s8}$ embryos have defective heart development. In comparison, 92% of zygotic mutant *s8* embryos have heart defects. The increase in penetrance of heart phenotypes to 100% in $MZtoh^{s8}$ embryos suggests that maternally deposited *toh* message or protein may be rescuing some gene function during heart development in zygotic *s8* mutant embryos.

3.III. *two of hearts* and *miles apart*

In addition to the obvious cardiac defects, *toh* mutants also display blistering in the tip of the tail (Figure 3.1D, arrowheads). The combination of the blister phenotype and cardia bifida is reminiscent of another zebrafish mutant, *miles apart (mil)* (Figure 3.1G and H; Kupperman et al., 2000). In this section I discuss the phenotypic similarities between the *toh* and *mil* genes and I show that *toh* is required for signaling via Miles Apart (Mil) but not the related receptor, $S1P_1$.

3.IIIa. *two of hearts* and *miles apart* mutants have identical phenotypes

Because of the similarities between the heart and tail phenotypes of *toh* and *mil* mutant embryos, I investigated whether or not these mutants share any other phenotypes. *mil* mutant embryos have previously been shown to have defects in the morphogenesis of the anterior endoderm (Kupperman et al., 2000).

I visualized the anterior endoderm of wild-type (Figure 3.1C), *toh* mutant (Figure 3.1F) and *mil* mutant (Figure 3.1I) embryos using transgenic zebrafish expressing green fluorescent protein (GFP) under the control of a fragment of the *her5* promoter known to drive expression in the anterior endoderm (Tallafuss and Bally-Cuif, 2003). In wild-type embryos expressing such a transgene, the anterior endoderm forms a cohesive sheet spanning the midline. However, embryos lacking *toh* or *mil* gene function display defective anterior endoderm morphogenesis. In embryos injected with a morpholino antisense oligonucleotide (MO) against the *toh* gene product (described below) or in embryos carrying *mil* mutations, the anterior endoderm has evident gaps between *her5::gfp* positive cells (Figure 3.1F and 3.1I, arrowheads).

Figure 3.1

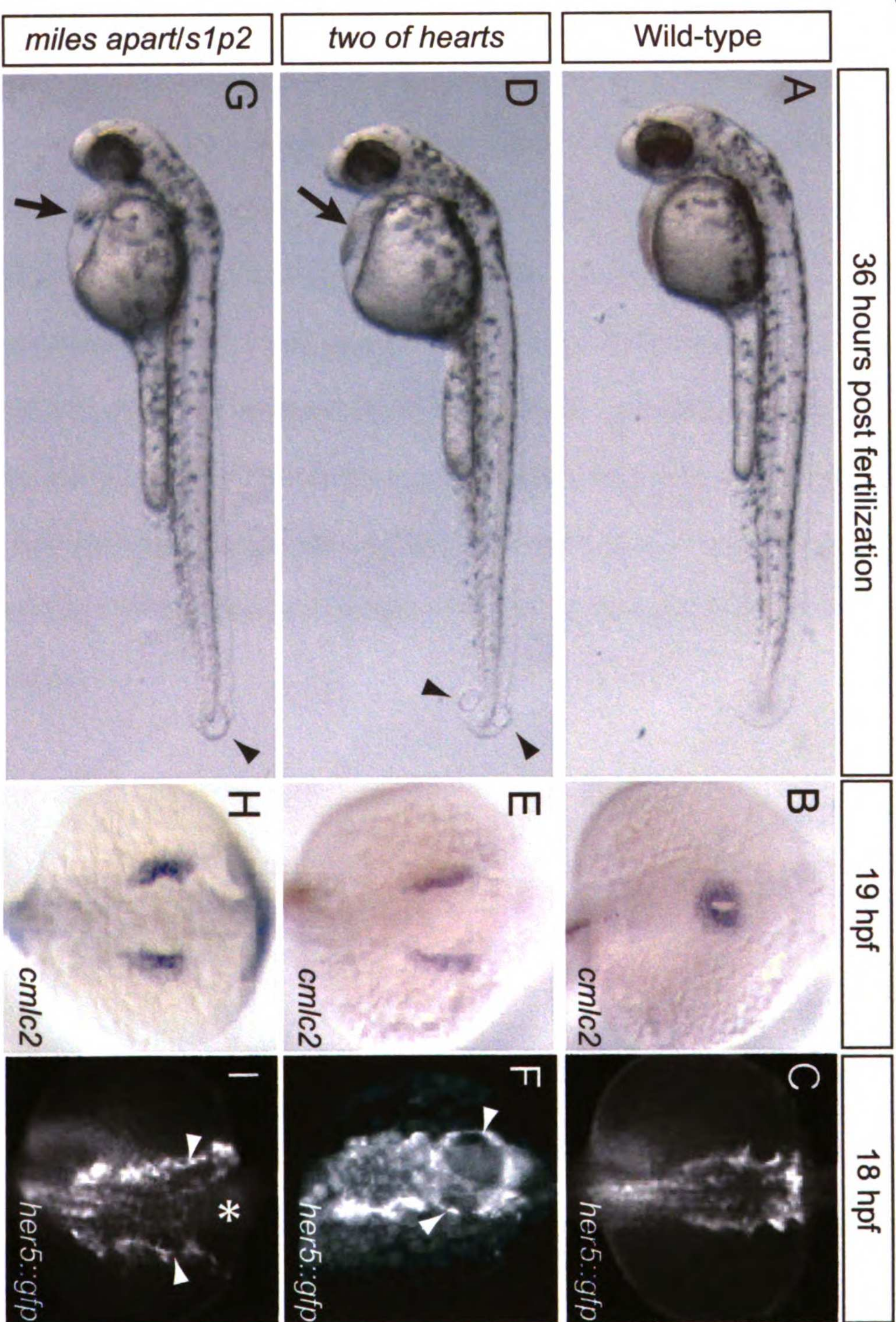


Figure 3.1: *two of hearts* and *miles apart* mutant phenotypes

Comparison of wild-type (A-C), *toh* (D-F), and *mil/s1p2* (G-I) mutant embryos. (A, D and G) Lateral brightfield images, anterior to the left, at 36 hpf show pericardial edema (arrow) and epidermal blisters (arrowheads) in the tails of *toh* (D) and *mil/s1p2* (G) mutant embryos. (B, E and H) Examination of *cmlc2* expression at 19 hpf shows heart-ring formation in wild-type embryos (B) and a failure in myocardial migration in *toh* (E) and *mil/s1p2* (H) mutant embryos. Dorsal views, anterior up. (C, F and I) Visualization of anterior endoderm by *her5::gfp* transgene expression at 18 hpf. In embryos injected with *toh* MO (F) and *mil/s1p2* (I) mutant embryos, the endoderm is irregularly shaped with numerous gaps (arrowheads) in the endodermal sheet. The most anterior region of *mil/s1p2* mutant embryos lack GFP positive endodermal cells at the midline (asterisk). Dorsal views, anterior up.

Although the defects in the endoderm of *toh* and *mil* mutants are similar, *mil* mutants seem to have more severe endoderm defects than those seen in cases of loss of *toh* function. Specifically, in *mil* mutant embryos the anterior most portion of the anterior endoderm is spread farther laterally and lacks GFP positive endodermal cells at the midline (asterisk). Embryos injected with *tohMO,* in contrast, have endodermal cells at the midline in that anterior region. This difference in severity of endoderm phenotypes is similar to differences in severity of heart phenotypes seen in *toh* versus *mil* mutant embryos. The failure in heart fusion in *mil* mutants is fully penetrant, while, as mentioned above, even the strongest allele of *toh* has some partial fusion of the heart fields. There is strong evidence that normal endoderm is required for the formation of the vertebrate heart tube (Alexander et al., 1999; Kikuchi et al., 2000; Komada and Soriano, 1999; Molkentin et al., 1997; Reiter et al., 1999). Based on the knowledge of the importance of the endoderm in heart morphogensis, I hypothesize that the heart phenotypes in *mil* and *toh* mutants are likely due to a primary defect in the morphogenesis of the anterior endoderm. The penetrance of cardia bifida is higher in *mil* than *toh* mutant embryos most likely because *mil* mutant embryos have a more severe anterior endoderm defect.

3.IIIb. *two of hearts* is required for signaling by Miles Apart/S1P$_2$

The *mil* locus is known to encode a G protein-coupled receptor (GPCR) that specifically responds to the lysophospholipid ligand sphingosine 1-phosphate (S1P) (Kupperman et al., 2000). *mil* specifically encodes an orthologue of the vertebrate S1P$_2$ receptor, previously called Edg5 (Kupperman et al., 2000; Osborne and Stainier, 2003). Because

toh mutants share phenotypes with *mil* mutants, I hypothesized that the *toh* locus might function in signaling through the S1P dependent Mil.

To test whether or not *toh* is required for signaling by Mil, I overexpressed the *mil* gene in wild-type and *toh* mutant embryos. Overexpression of *mil* RNA by injection into the single celled embryo has profound effects on zebrafish development. Wild-type embryos overexpressing *mil* show severe morphological defects at 30 hpf (89%, n = 208; Figure 3.2A). These defects include cyclopia (Figure 3.2A, asterisk), shortened body axis and disorganization of the somitic mesoderm. Many of the defects resulting from *mil* overexpression can be traced to defects in cell movements during gastrulation. In fact, embryos overexpressing *mil* show defects in both convergent extension and ebiboly movements during gastrulation (Figure 3.2D). The defects in convergent extension movements result in widened axial mesoderm (solid bar), visualized by expression of the *foxA2* gene. Epiboly defects result in slower progress of the leading edge of gastrulation towards the vegetal pole of the yolk cell (dashed bar). In some embryos the delay in epiboly movements is so severe that the yolk cell is never entirely enclosed by embryonic tissue. This failure in completion of epiboly is lethal to the embryo as it results in rupture of the yolk cell or exogastrulation.

The severe early defects seen in *mil* overexpressing embryos allow us to test whether Mil mediated signaling is intact in *toh* mutant embryos. To test this question, I injected *mil* RNA at levels known to disrupt wild-type gastrulation movements into *toh* mutant embryos or into embryos co-injected with *tohMO*. Unlike wild-type embryos, only 7% of *toh* mutants (n = 208 embryos) show morphological defects at 30 hpf when overexpressing *mil* (Figure 3.2B). In fact, *toh* mutant embryos overexpressing *mil*

appeared almost completely morphologically normal, with the notable exception that these embryos still display pericardial edema (Figure 3.2B, arrow) and blistering in their tails (arrowheads). The presence of heart defects and tail blisters in these embryos suggests that overexpression of *mil* is incapable of rescuing *toh* loss of function. Looking during gastrulation I found that, as expected, embryos with decreased *toh* function show little or no gastrulation defects when overexpressing *mil* (Figure 3.2E). Therefore, loss of *toh* function rescues embryos from the effects of inappropriate signaling via the Mil receptor.

The *mil* overexpression data support two conclusions. The first conclusion is that the function of the *toh* locus is required for exogenous Mil receptor to affect embryonic development. Secondly, overexpression of *mil* RNA cannot rescue the *toh* mutant phenotype. The first conclusion, combined with the phenotypic commonalities between *toh* and *mil* mutant embryos, supports the hypothesis that *toh*, indeed, is required for the Mil signaling pathway. While these data do suggest that *toh* is required for signaling via the Mil receptor, it is not clear from this experiment at what level of Mil mediated signaling *toh* is acting. It is also not clear if *toh* is specific to signaling by Mil alone or if, perhaps, *toh* affects several signaling pathways.

3.IIIc. *miles apart* overexpression is sphingosine 1-phosphate dependent

One mechanism by which *toh* mutations might affect *mil* signaling would be through control of the availability of S1P, the ligand for Mil. In order to test this hypothesis I first needed to determine whether overexpressed *mil* RNA was signaling in an S1P dependent manner. To verify this dependency, I generated a single amino acid change in the Mil

Figure 3.2

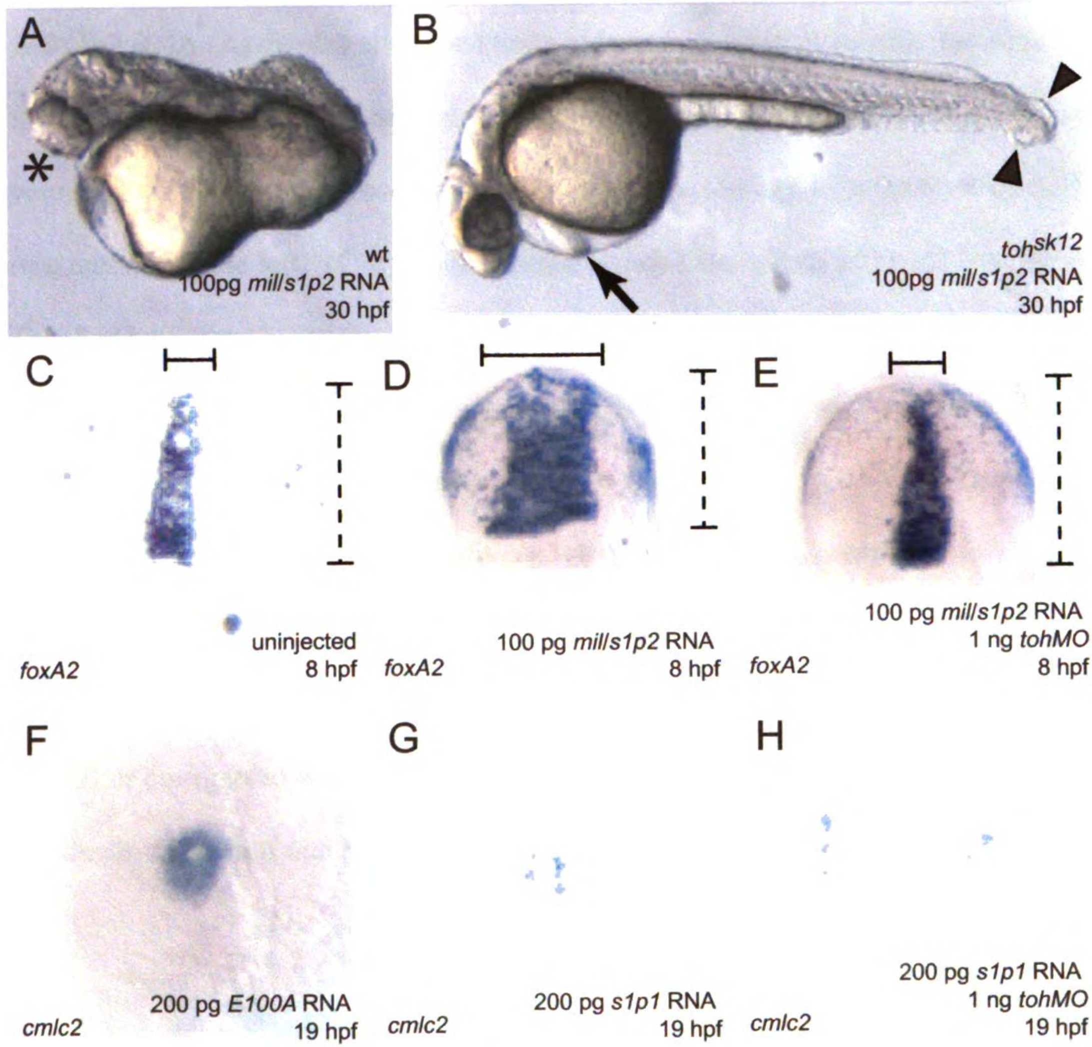


Figure 3.2: *two of hearts* function is required for $S1P_2$ but not $S1P_1$ mediated signaling

(A and B) embryos overexpressing *mil/s1p2* at 30 hpf. Wild-type embryos injected with 100 pg of *mil/s1p2* RNA (A) display shortened body axis and cyclopia (asterisk). *toh* mutants injected with 100 pg of *mil/s1p2* RNA have no body axis defects (B) but develop pericardial edema (arrow) and tail blistering (arrowheads), phenotypes seen in uninjected *toh* mutants. Lateral views, anterior to the left. (C-E) Visualization of axial mesoderm and endoderm by expression of *foxA2* at late gastrula stages. Wild-type embryos overexpressing *mil/s1p2* (D) have broadened axial mesoderm (solid line) when compared to uninjected embryos (C). Extent of epiboly (dashed line) is shorter in embryos overexpressing *mil/s1p2* as compared to uninjected siblings. *toh* mutant embryos overexpressing *mil/s1p2* (E) are indistinguishable from uninjected siblings. Dorsal views, animal pole up. (F-H) *cmlc2* gene expression at 19 hpf. Embryos injected with 200 pg of *s1p1 E100A* RNA (F) have normal *cmlc2* expression. Embryos injected with *s1p1* RNA (G) or co-injected with *s1p1* RNA and *toh* MO display decreased *cmlc2* expression levels likely due to decreased number of myocardial cells.

protein that has previously been shown to disrupt interaction between S1P receptors and S1P (Parrill et al., 2000). RNA from this *mil E129A* construct resulted in no detectible phenotypes when injected at either 100 pg or 200 pg of RNA per embryo (not shown). This lack of overexpression phenotypes with *mil E129A* contrasts with the overexpression phenotypes seen with wild-type *mil* RNA and suggests that S1P interaction with Mil is critical in the generation of overexpression phenotypes. Because the overexpression of *mil* requires an interaction with S1P, *toh* might be functioning in the production or release of S1P in the embryo.

3.IIId. *two of hearts* is not required for the overexpression phenotype of *s1p1*

Exploring the possibility that *toh* might be required for S1P production or release, I then overexpressed another S1P receptor, *s1p1*(Im et al., 2000), in wild-type and *toh* mutant embryos. In addition to the wild-type *s1p1* construct, I generated a version of *s1p1*, *s1p1 E100A,* containing a mutation similar to that used to verify that *mil* requires S1P interaction to signal under overexpression conditions.

Two phenotypes are seen in embryos overexpressing wild-type *s1p1*. First, embryos sometimes display shortened and disorganized body axes. Interestingly, the body axis phenotype in *s1p1* overexpressing embryos is not dependent on interaction with S1P, as equivalent numbers of embryos overexpressing *s1p1 E100A* also have similar shortened and disrupted body axes. This S1P independent phenotype suggests the possibility that the zebrafish $S1P_1$ may have some ligand-independent activity.

The second phenotype seen was that 86% of wild-type embryos injected with RNA encoding *s1p1* displayed reduction or loss of myocardial tissue, as determined by

expression of *cmlc2* (Figure 3.2G; n = 110 embryos). In embryos allowed to develop further, the decrease in myocardial tissue led to small, non-functional hearts or, in more severe cases, cardia bifida (not shown). Unlike the effects on the body axes, this myocardial phenotype is dependent upon receptor interatction with S1P as only 4% of embryos injected with *s1p1 E100A* were scored as having decreased *cmlc2* expression by in situ hybridization (Figure 3.2F; n = 45 embryos).

When embryos were coinjected with *s1p1* RNA and *tohMO* (Figure 3.2H), 81% of the embryos display a decrease in *cmlc2* expression (n = 93 embryos), suggesting that loss of *toh* function is not sufficient to rescue overexpression of *s1p1*. Therefore, although loss of function of the *toh* gene is sufficient to rescue embryos from the effects of S1P dependent Mil signaling, it is not sufficient to protect embryos from S1P dependent signaling downstream of the *s1p1* gene. If *toh* mutations resulted in limited S1P production or release, it would be expected that *toh* mutations would be capable of rescuing overexpression of other S1P dependent receptors. Therefore, it is unlikely that *toh* is affecting Mil signaling by limiting the available S1P.

Furthermore, these *s1p1* overexpression experiments also indicate that it is unlikely that *toh* gene function is required for signaling by all GPCRs. The concept that *toh* is specific to the *mil* pathway or to a limited subset of GPCRs is further indicated by the fact that processes known to require GPCR signaling are unaffected in *toh* mutant embryos. One such process unaffected in *toh* mutants is the migration of the posterior lateral line sensory primordium (not shown), a process known to require signaling via GPCRs of the CXCR4 family (David et al., 2002b; Li et al., 2004). It is, therefore, possible that *toh* may be required only in the specific case of signaling via the *mil*

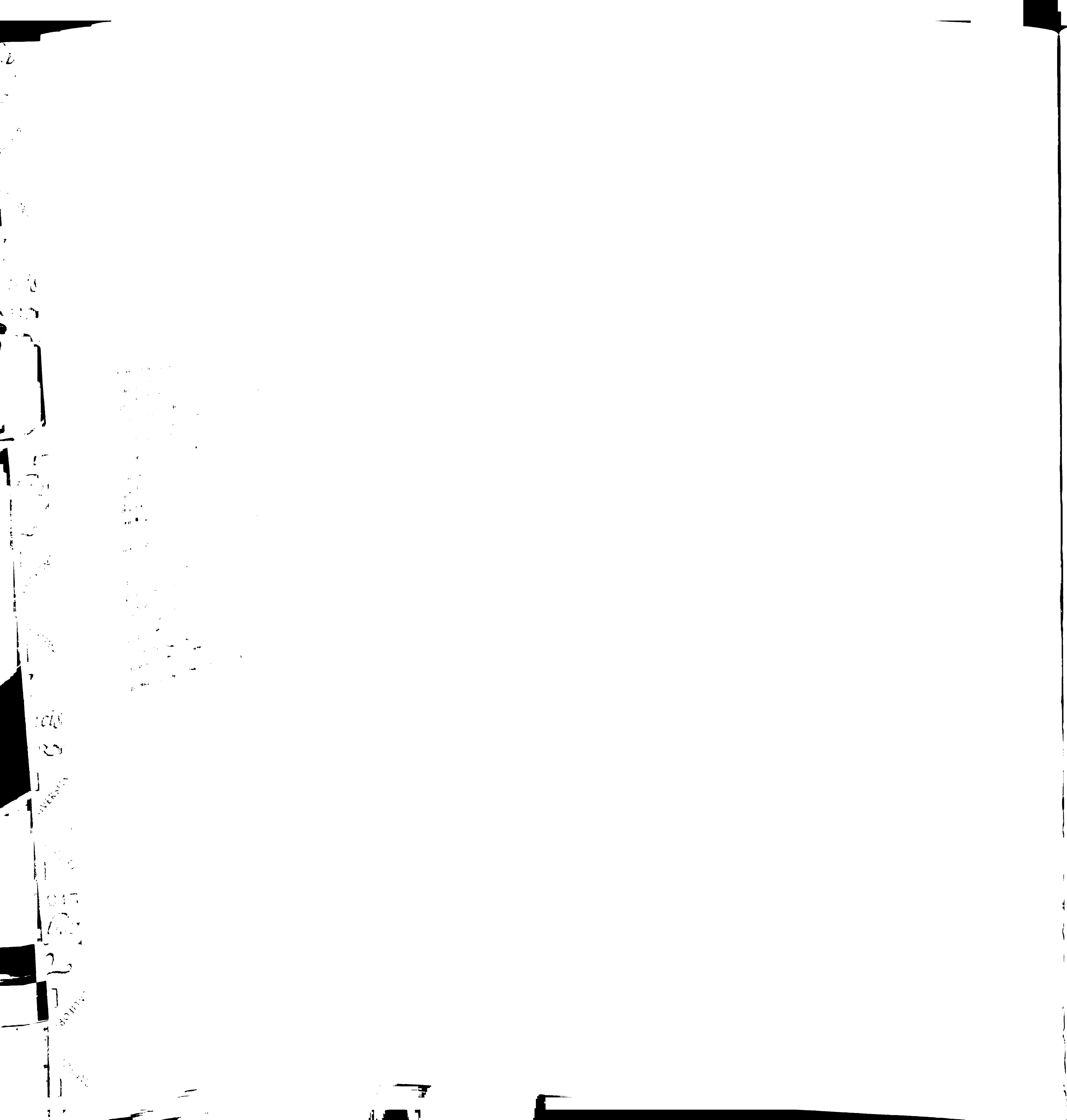
pathway. However, it will require further investigation to determine if *toh* is, in fact, specific to a Mil signaling or affects more than one signaling pathway.

3.IV. Identification of the *two of hearts* locus

While I sought understand how the *toh* locus might play a role in the Mil signaling pathway, I also wanted to determine the identity of the gene affected in the four *toh* alleles. To do so I undertook the positional cloning of the *toh* locus. In this section I discuss the mapping of the *toh* locus, the identification of *toh* as a *spinster-like* gene, the nature of the lesions in the *s8, sk12* and *s420* alleles and the expression pattern of *toh.*

3.IVa . Mapping *two of hearts*

Using bulk segregant analysis I localized the *toh* locus to zebrafish chromosome 5 (Figure 3A). The process of fine mapping used 1197 diploid *s220 toh* mutant embryos to narrow the affected locus to a 295 kilobase region between the CA repeat marker z9419 and a single nucleotide polymorphism (SNP) in a homologue of the mammalian *ankhzn* gene. This SNP was designated ankdCAP4 (see Materials and Methods). The 295 kilobase region is covered entirely by three bacterial artificial chromosomes (BACs): CHORI-211 134D21, CHORI-211 138A6 and DanioKey 7B17. These BACs have been sequenced and assembled by the Sanger Centre *Danio rerio* Sequencing Project. Full length sequences of these BACs are available at ftp://ftp.sanger.ac.uk/pub/sequences/zebrafish. Within the critical genetic region between z9419 and ankdCAP4 were portions of six putative open reading frames (ORFs). These ORFs were defined using a combination of the GENSCAN exon prediction software



(Burge and Karlin, 1997) and analysis of conservation of synteny between zebrafish, mouse and human genomic sequences.

3.IVb. The *two of hearts* locus encodes a *spinster-like* gene

In three of the four *toh* alleles, lesions were found in one embryonically expressed gene (Figure 3.3A, gray filled open arrow). This gene encodes a putative 12 pass transmembrane domain protein of the major facilitator superfamily (MFS) of non-ATP dependent transporters (Figure 3.3B). The protein is predicted to be 504 amino acids in length and is related to the *spinster-like* family of genes (Figure 3.4). The *spinster-like* family of genes is named after the *Drosophila spinster* gene, also called *benchwarmer* (Dermaut et al., 2005; Sweeney and Davis, 2002; Yamamoto and Nakano, 1999).

In the *s8* allele of *toh*, a T→G transversion at the second nucleotide of Intron 9 disrupts a splice donor site resulting in read through of intronic sequence. Contained within this intron are several in-frame stop codons leading to predicted truncation of the protein created from this message (Figure 3.3B). In the *sk12* and *s420* alleles, independent mutations affect the splice acceptor site between Intron 1 and Exon 2. In *sk12* this is the result of a G→C transversion at the Exon 2 +8 nucleotide position. In *s420* a nearby A→T transversion in Intron 1 (Exon 2 -2 nucleotide position) disrupts the splice acceptor site. The resulting mRNA from these alleles yields a predicted protein with a fusion of the first and second transmembrane domains and a deletion of the intervening loop (Figure 3.3B). No lesion was found in the coding sequence of the *s220* allele, suggesting that the lesion may be in a non-coding region of the gene.

Figure 3.3

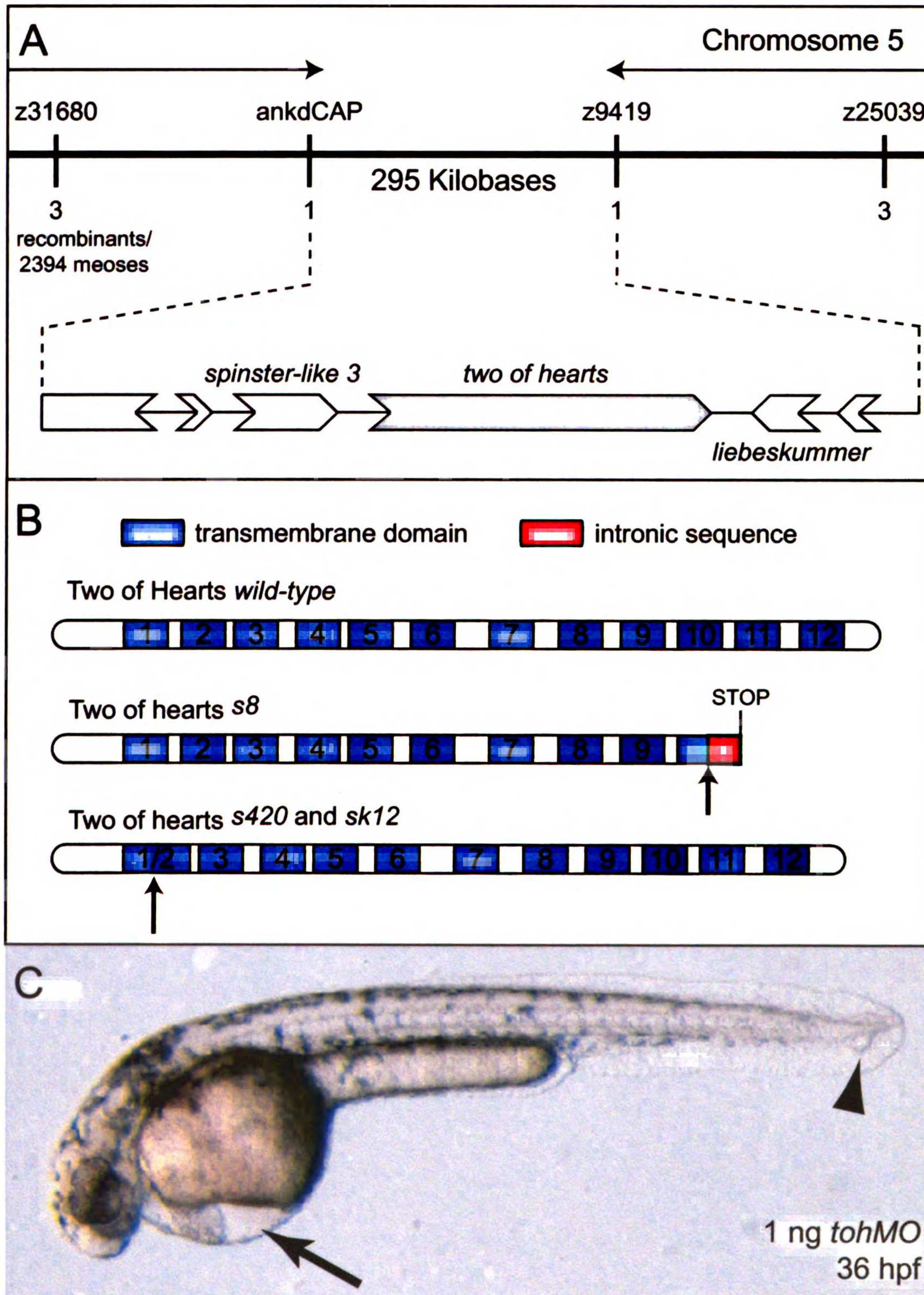


Figure 3.3: Identification of the *two of hearts* gene

(A) Positional cloning of *toh*. Direction of the chromosomal walk is indicated with black arrows above the marker names. Markers used in mapping are indicated above the line representing the genomic region. The numbers of recombination events out of 2394 meioses found at each marker are indicated below the genomic region. A blow up of the critical region is shown. Portions of 6 open reading frames (blowup, open arrows) are found in the identified genetic interval, including the previously cloned locus *liebeskummer* gene. The critical region contains two *spinster-like* genes, one of which was identified as *toh* (gray filled arrow) and the other we name here *spinl3*. (B) *toh* alleles diagrammed schematically with amino acid start and end positions indicated below each representation. Arrows indicate the site affected in the mutant alleles. Transmembrane domains are indicated in blue and the predicted translated intronic sequence in the *s8* allele is indicated in red. Numbers identify the transmembrane domains starting from the N-terminus. (C) Injection of a splice blocking MO against *toh* phenocopies effects of *toh* mutations, causing pericardial edema (arrow) and blistering in the tail (arrowhead).

3.IVc. Verification of the *two of hearts* locus by rescue and loss of function

To verify that this *spinster-like* gene is the *toh* locus, I then carried out rescue and loss of function experiments. To test for rescue, the putative *toh* mRNA was injected into *MZtoh*^{s8} embryos and into zygotic *s420* embryos. In both *MZtoh*^{s8} and zygotic *s420* embryos, injection of the *spinster-like* gene rescued PCM migration in some mutant embryos (not shown). *toh* mRNA injection rescued circulation in *s420* mutants, allowing these embryos to be raised to adulthood. In contrast to overexpression of the putative *toh spinster-like* gene, heart development in *toh* mutants is not rescued by overexpression of RNA encoding *Drosophila spin* or either of the two other *spinster-like* genes found in the fish genome, *not really started (nrs) (*Young et al., 2002) and a gene I have name *spinster-like 3 (spinl3).*

To further verify that this *spinster-like* gene is the *toh* locus, I carried out complementary loss of function experiments. Loss of function was achieved using a MO that blocks the splice donor site between Exon 4 and Intron 4 of the putative *toh* RNA. Embryos injected with this MO displayed the phenotypes seen in *toh* mutants, including cardia bifida and tail blisters (Figure 3.3C). Therefore, *toh* rescue and loss of function data, combined with the lesions found in the three strongest alleles of *toh,* indicate that the *toh* locus encodes this *spinster-like* gene.

3.V. *two of hearts* is ubiquitously expressed

Having identified the affected locus in *toh* mutants, I then examined the expression pattern of the *toh* gene. Although *toh* mutants have defects in specific tissues, and at specific times in development, the *toh* gene is expressed ubiquitously (not shown).

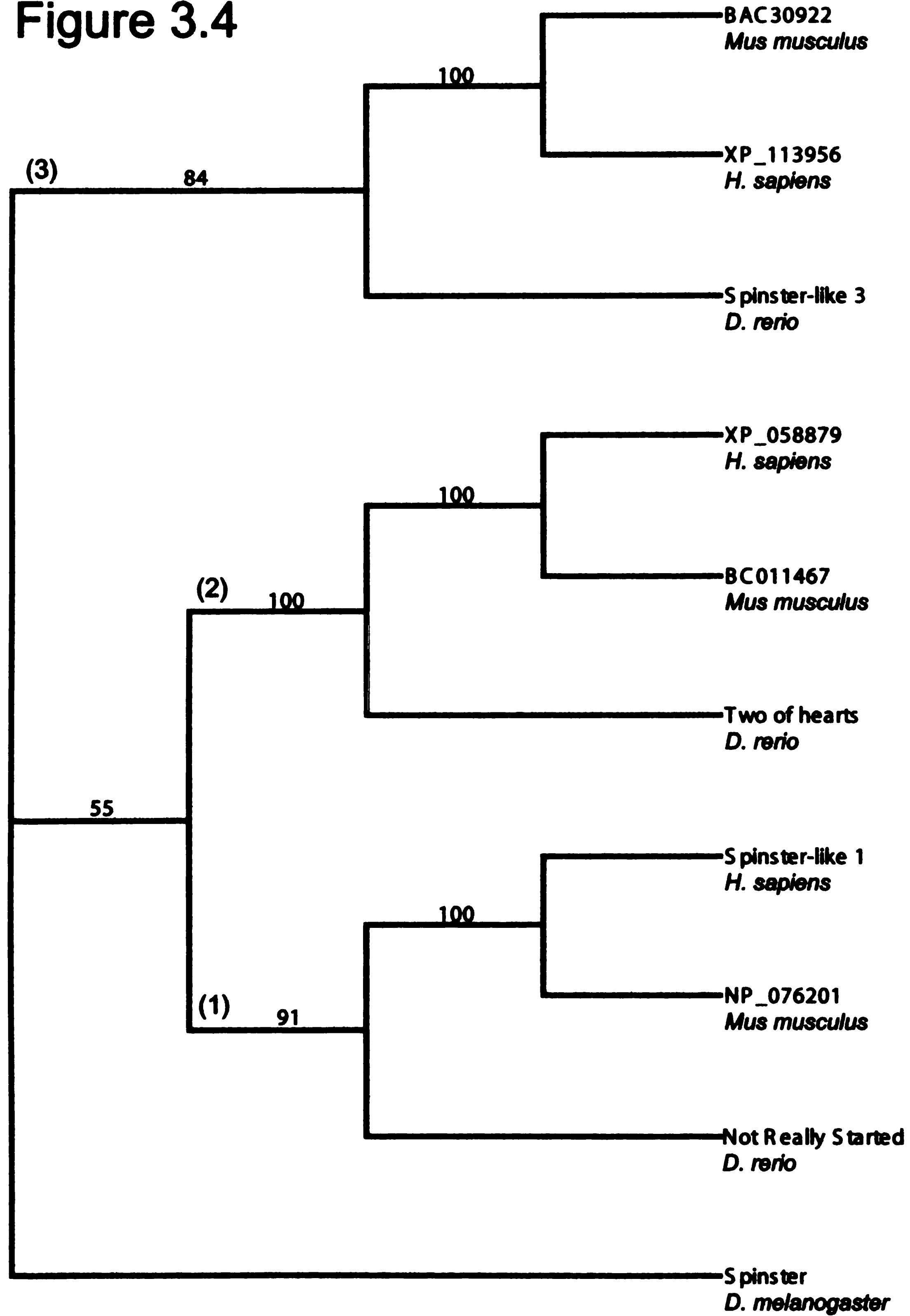


Figure 3.4
100
(3)
84
BAC30922
Mus musculus
XP_113956
H. sapiens
Spinster-like 3
D. rerio
100
XP_058879
H. sapiens
(2)
100
BC011467
Mus musculus
Two of hearts
D. rerio
55
100
Spinster-like 1
H. sapiens
(1)
91
NP_076201
Mus musculus
Not Really Started
D. rerio
Spinster
D. melanogaster

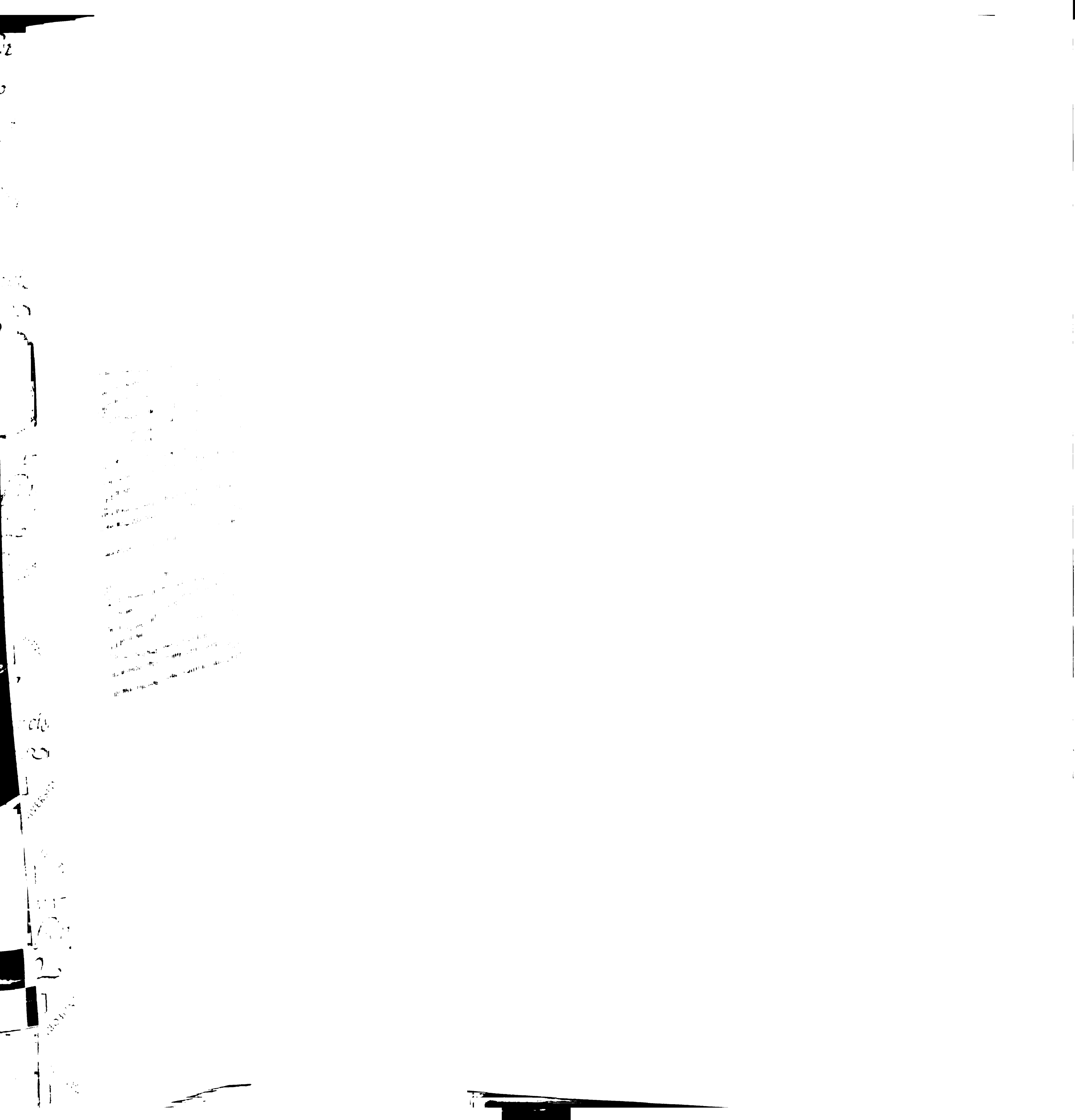


Figure 3.4: Phylogenetic analysis of the vertebrate Spinster proteins

Proteins are identified by name or by GenBank Accession Number. Alignments were carried out using ClustalX and phylogentic analysis was done with PAUP* 4.0b10 software, using maximum parsimony as the optimality criterion, starting with 100 random addition sequences and tbr branch-swapping. 1000 bootstrap pseudoreplicates were performed. Bootstrap support values are indicated at branch points. Three monophyletic clades are hypothesized, (1) Spinster-like 1, (2) Spinster-like 2 and (3) Spinster-like 3. Zebrafish Two of Hearts belongs to the Spinster-like 2 family of orthologues, while Not Really Started is most closely related to Human Spinster 1 and the Spinster-like 1 group. Two of Hearts and zebrafish Spinster-like 3 were sequenced from zebrafish cDNA and all other sequences were downloaded from GenBank.

Embryos examined by in situ hybridization against the *toh* gene show uniform expression in all tissues from 8-cell stage (1.25 hpf) to 48 hpf.

The fact that this gene is ubiquitously expressed, but affects only certain cells might seem to be difficult to reconcile. However, as has been shown above, one of the main, if not only, functions of the *toh* genes is to mediate signaling by the *mil* gene pathway. Unlike *toh, mil* is expressed in a much more restricted pattern (Figure 2.2; Kupperman et al., 2000). At the stages when the PCM cell populations are migrating, *mil* is specifically expressed in the anterior endoderm, the midbrain-hindbrain boundary and the tip of the tail. Therefore, even though *toh* is expressed throughout the embryo, its function may be restricted to tissues that express its signaling partner, *mil.*

3.VI. Subcellular effects and localization of *two of hearts*

The most fundamental question regarding *toh* is how this putative transporter functions in signaling by a GPCR. What little data that exists for *spinster-like* genes, as was mentioned above, suggests that these genes may control aspects of cell death regulation and subcellular trafficking. In *Drosophila* the role of *spin* in subcellular trafficking seems be required for the exclusion of sugars and lipids from the lysosomal compartment of the cell. *spin* mutants develop inappropriate accumulation of these lipids and sugars in the lysosome (Dermaut et al., 2005; Nakano et al., 2001; Sweeney and Davis, 2002). One of the consequences of this inappropriate accumulation of lipids and sugar moieties in the lysosome is an expansion of that cellular compartment. Because of the cellular effects of *spin* mutations, I wanted to test whether *toh* might have a similar role in the control of intracellular trafficking in zebrafish.

In this section I present evidence that *toh* does not affect the size of the lysosomal compartment or the gross structure of the recycling endosome. In addition I demonstrate that Toh localizes in a subcellular compartment and can, on rare occasions be found in the same or immediately adjacent compartments with Mil.

3.VIa. Loss of *two of hearts* function does not cause lysosomal expansion

One mechanism by which *toh* might affect *mil* would be to affect trafficking of the receptor. The lysosomal defects in *Drosophila spin* mutants suggest a mechanism for disrupting receptor trafficking in cells. Therefore, in order to test whether or not *toh* mutant embryos have similar lysosomal expansion to the *Drosophila spinster* mutants, I examined the size of the acidified compartment of cells in wild-type (Figure 3.5A) and *tohMO* injected (Figure 3.5B) embryos. The acidified cellular compartments are equivalent between wild-type and *tohMO* injected embryos, suggesting that *toh,* unlike *Drosophila spin*, does not regulate lysosomal size. In addition to *tohMO* injected embryos, *MZtoh*^{s8} embryos also are indistinguishable from wild-type embryos with respect to the size of their acidified cellular compartment (not shown).

3.VIb. The recycling endosome appears unaffected by loss of *two of hearts* function

If *toh* is affecting the trafficking of *mil* it may function in a similar fashion to *Drosophila spin* but in an earlier cellular compartment in the endocytic pathway. Therefore, I visualized the early and recycling endosomal compartments of embryos using a yellow fluorescent protein (YFP) tagged zebrafish Rab5c protein (Ulrich et al., 2005). Rab5 small GTPases are known to be targeted to the early and recycling compartments of the

endocytic pathway, where they regulate membrane fusion events (Barbieri et al., 1996). Rab5c-YFP localization in wild-type (Figure 3.5C) and *tohMO* injected (Figure 3.5D) embryos is indistinguishable, suggesting that *toh* is not affecting this cellular compartment, either. Therefore, examination of lysosomal staining and Rab5c-YFP localization in *toh* mutant, or MO injected, embryos suggest that *toh* is not affecting *mil* signaling by globally affecting either the early or late compartments of the endocytic pathway. Although it is still possible that *toh* affects some aspect of the mechanics of intracellular trafficking, it seems that if this is the case, *toh* functions in a fashion with less obvious effects than those of the *Drosophila spinster* gene.

3.VIc. Two of Hearts localizes to an intracellular compartment

As *toh* seems to be fairly specifically required for signaling by the *mil* gene product, understanding the physical relationship between the localization of Toh and Mil might suggest how these proteins interact. In order to determine the subcellular localization of Toh, I generated a C-terminal fusion of Toh with the myc-epitope tag and injected embryos with RNA encoding this fusion protein (Figure 3.5E). Toh appears to be primarily localized to an intracellular compartment. I compared Toh-myc distribution to that of an HA tagged Mil construct (Figure 3.5F). HA-Mil also localizes to an intracellular compartment. The pattern of HA-Mil localization is consistent with an actively signaling and, therefore, internalized GPCR. When the images of Toh-myc and HA-Mil localization are merged (Figure 3.5G and G') one can see that the majority of Toh-myc and HA-Mil signal do not co-localize in the cell.

Figure 3.5

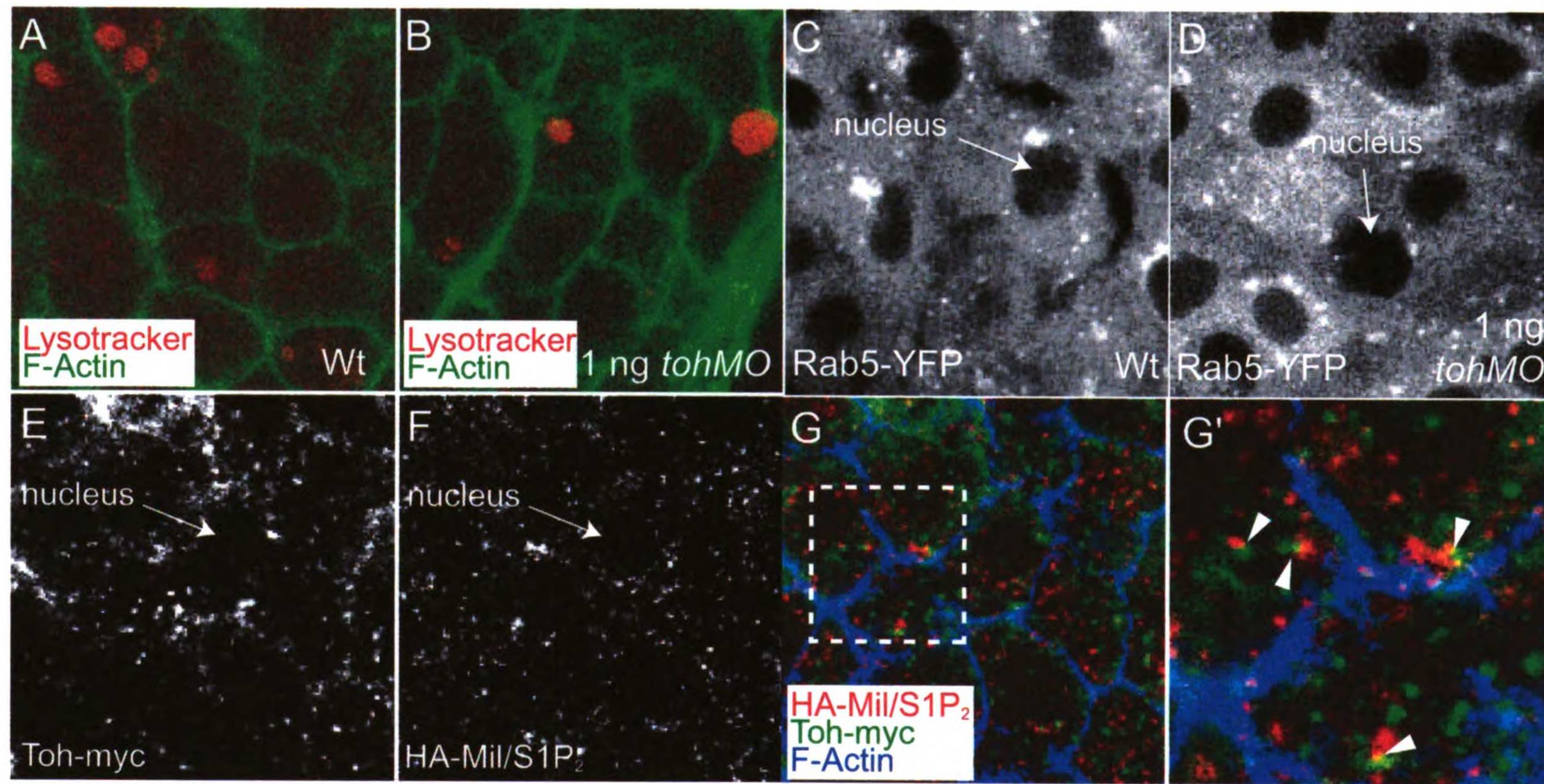


Figure 3.5: Cellular effects of *two of hearts*

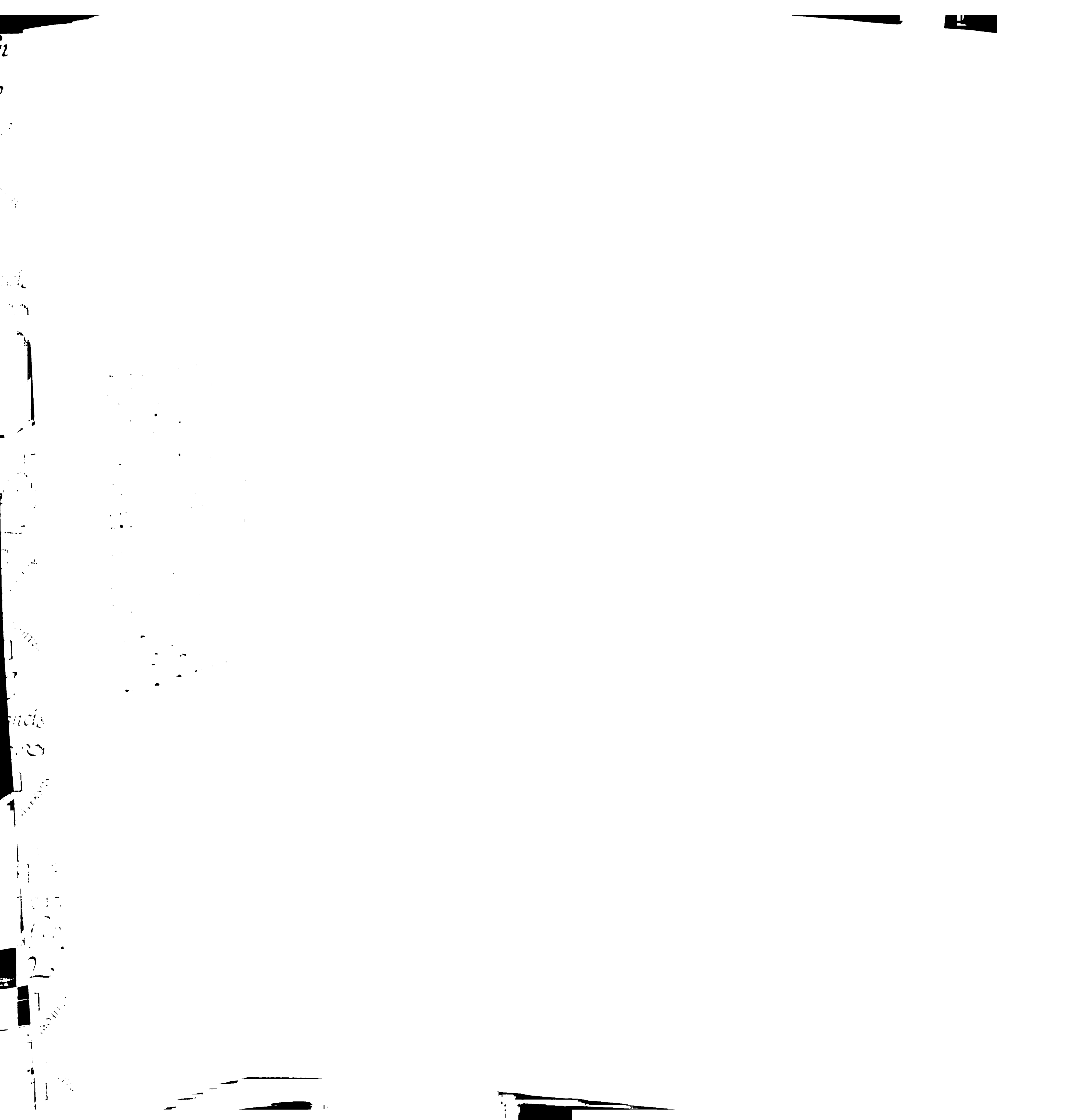
(A and B) Confocal analysis of embryos stained with lysotracker DND-99 (red), counterstained for F-actin (green), and imaged 8 hpf . Wild-type embryos (A) and *tohMO* injected embryos (B) show similar levels of staining (C and D). Rab5c-YFP localization 8 hpf in wild-type embryos (C) and *tohMO* injected embryos (D) also appears similar. (E, F, G and G') Subcellular localization of *myc*-tagged Toh (E) and HA-tagged Mil/S1P_2 (F) 8 hpf. The merged image (G and G') counterstained for F-actin (blue), shows that HA-Mil/S1P_2 (red) and Toh-myc (green) sometimes appear to localize in adjacent or slightly overlapping compartments (yellow; arrowheads). Panel (G') is a blow up of a portion of panel (G) (box).

In a minority of cases, however, the Toh-myc and HA-mil signals do appear to co-localize (Figure 3.5G', arrowheads). When Toh-myc and HA-Mil appear to co-localize, however, they never completely overlap, suggesting that if they are in the same subcellular compartment they may be partitioned to different regions of that compartment. It is also possible that the two proteins are localized to adjacent structures within the cell giving the false impression of overlap. However, the close proximity of some pools of these proteins does suggest the possibility that Toh may interact with Mil physically and may affect its signaling via such an interaction.

Given the possibility that Toh is a small solute transporter, however, physical interaction between Toh and Mil may be unnecessary for signaling to take place between these proteins. Instead, Toh may function to modify the subcellular compartments in which Mil is found. This modification might be achieved by changing the intracellular localization of internalized Mil itself or by modifying the characteristics of the compartments into which Mil is trafficked. Either of these cases would be predicted to affect Mil signaling. While I have been unable to find any evidence for large scale change of Mil localization within the cells of *toh* mutant embryos (not shown), I cannot exclude the possibility that there are changes in Mil trafficking due to *toh* mutations.

3.VII. Perspectives and future directions

I have identified *toh* as a novel component involved in signaling via the zebrafish $S1P_2$ orthologue, Mil. The combination of shared phenotypes between *toh* and *mil* mutants and the fact that loss of *toh* function rescues embryos from the deleterious effects of *mil*

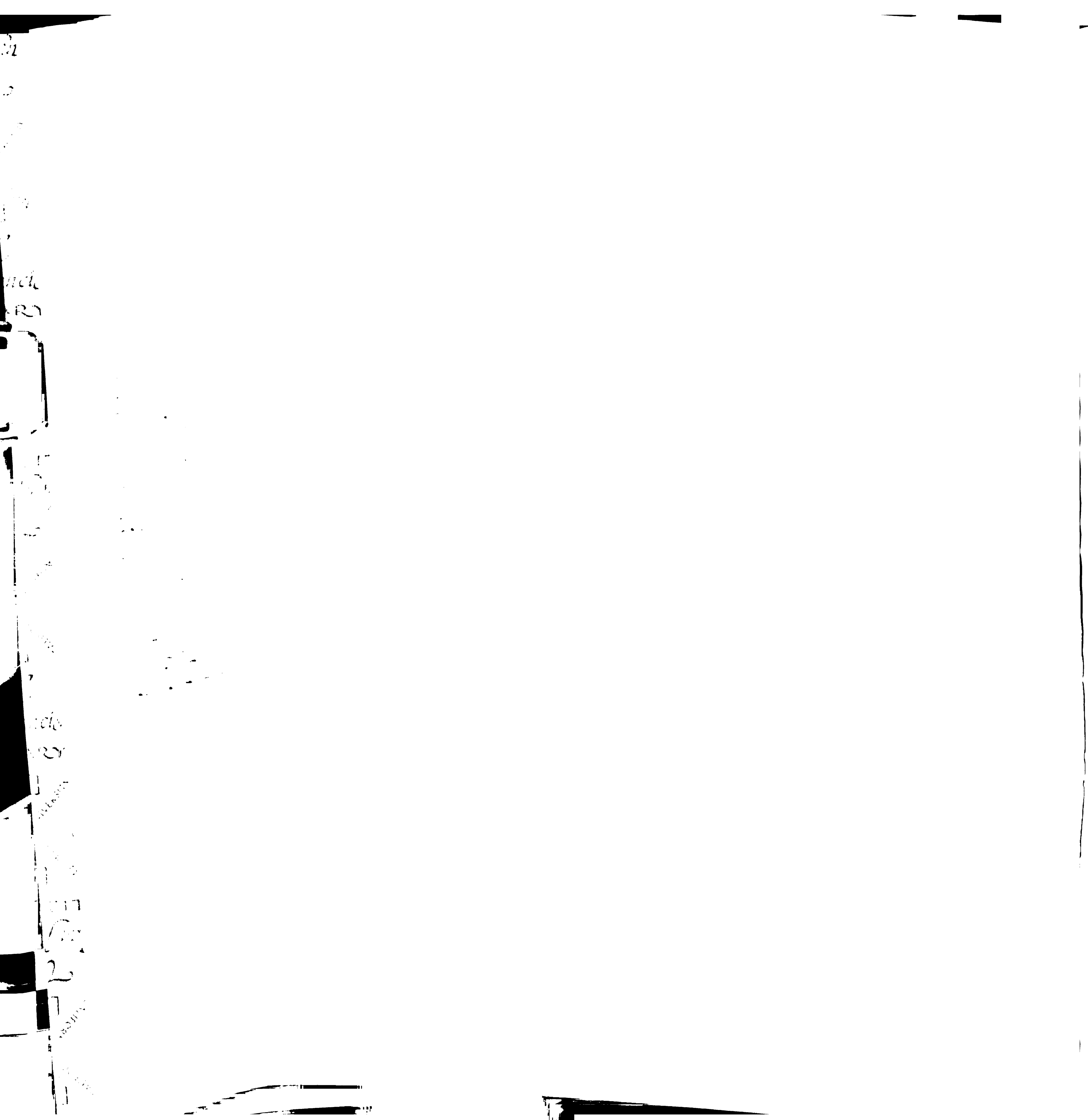


overexpression suggest that *toh* is a necessary component of this signaling pathway. Furthermore, while I cannot verify that *toh* affects only *mil* signaling, I have shown by overexpression of *s1p1* that the function of *toh* is not required by all GPCRs or even all S1P receptors. The idea that *toh* function is not required for signaling by all GPCRs is also supported by the fact that other developmental processes known to require GPCR signaling are unaffected in *toh* mutant embryos.

In addition, I have identified the gene affected in *toh* mutant embryos as a novel zebrafish homologue of the *Drosophila* gene *spin.* Although there is evidence that TGFβ signaling in *Drosophila* is epistatic to mutations in *spin* (Sweeney and Davis, 2002), this study provides the first evidence for a direct link between a specific signaling pathway and a *spinster-like* gene.

Fundamentally, however, the question of how *toh* affects signaling via Mil has not been answered. In part this is due to a limited understanding of the biochemical function of the *spinster-like* genes. Knowledge of what small solutes, if any, are transported by Toh would help in understanding the relationship between Toh and Mil. However, no data exist that definitively identify the target molecule or molecules transported by any Spinster-like proteins.

As was stated above, recent studies have suggested that the expanded lysosomal compartments in *spin* mutant flies contain inappropriate accumulations of polysaccharides in addition to lipid accumulation (Dermaut et al., 2005). This accumulation of sugar moieties in the lysosomal and late endosomal compartments of *spin* mutants might suggest that this gene is a polysaccharide transporter. Sequence homology between the *spinster-like* genes and sugar transporters also support this



hypothesis. However, sequence homology is a poor predictor of MFS transporter targets, as MFS proteins that are closely related by sequence homology can have vastly different transport substrates (reviewed in Pao et al., 1998). Another consequence of homology being of questionable use in determining the molecules transported by MFS proteins is that that *Drosophila* Spin and Toh may have evolved the capacity to transport vastly different solutes.

The mechanism that may have allowed the solutes transported by Spin and Toh to have evolved in divergent directions is the radiation of the *spinster-like* genes that occurred in the vertebrate lineage, but not in arthropods. Vertebrate genomes contain at least three *spinster-like* genes, in contrast to *Drosophila* which has only one *spinster-like* gene. As a result of the duplication of *spinster-like* genes, these genes may have diverged in both sequence and in function. This idea is supported by the fact that the human *spinster-like 1* and the zebrafish *nrs* gene both apparently have the ability to regulate cell death at some level. In contrast, there is no evidence of cell death effects from either loss or gain of *toh* function (not shown). Therefore, it is likely *toh*, and potentially its mammalian orthologue, has evolved a novel function that is divergent from other *spinster-like* genes. Finally, it should be noted that the possibility exists that the divergent function of the Toh protein may, in fact, not involve transport of solutes at all.

Available data suggests that *toh* is somewhat specific to Mil signaling. I have shown that another S1P receptor, $S1P_1$, signals independently of *toh* function, suggesting that *toh* does not act upstream of Mil by affecting S1P availability. Because *toh* is not involved in the production or export of S1P upstream of Mil, the possibility exists that *toh* acts downstream of Mil. Because Mil is a GPCR, signaling by this receptor would be

expected to involve the heterotrimeric G proteins. It is not known which specific G protein family members couple with Mil in zebrafish. However, mammalian $S1P_2$ is known to couple to G_i, G_{12}, and G_q family G proteins (Windh et al., 1999). If G protein coupling is conserved between Mil and mammalian $S1P_2$ receptors, these would be the G protein families whose signaling might be affected by *toh*. G_i signaling is an unlikely candidate to be affected by *toh*, as this is the sole G protein family that couples to mammalian $S1P_1$ (Windh et al., 1999) and zebrafish $S1P_1$ signals independently of *toh*. I also suspect that signaling by the G_{12} family cannot be entirely dependent on *toh* function. The reason for this suspicion is that members of the G_{12} family of G proteins are required for gastrulation movements in the zebrafish embryo (Lin et al., 2005) and I see no obvious gastrulation defects in *toh* mutants. However, there is some suggestion that *toh* does partially affect the ability of zebrafish $G\alpha_{12}$ to signal (not shown; see Appendix A). These experiments involved overexpression of a constitutively active version of $G\alpha_{12}$, a potent signaling molecule with numerous downstream effectors. Therefore, it is difficult to interpret these results, although these data may suggest that *toh* is, in fact, downstream of Mil and $G\alpha_{12}$.

As the relevance of S1P signaling in both basic and clinical sciences becomes more evident, it is critical that the signaling partners of S1P receptors are identified. The identification of *toh* as a component of $S1P_2$ signaling in zebrafish suggests that the mammalian orthologues of *toh* may have similar functions in $S1P_2$ mediated signaling. $S1P_2$ signaling has been shown to play critical roles in the activation of mast cells (Jolly et al., 2004), a cell type thought to contribute to the pathogenesis of asthma (Chiappara et al., 2001). In addition $S1P_2$ receptor function is known to affect vascular tone (Ohmori et

al., 2003), and may contribute to protective effects S1P has against injury from ischemic challenge to the heart during myocardial infarction (Karliner, 2004). Therefore, *toh* and its orthologues may represent new targets for the manipulation of specific S1P signaling pathways in both normal and pathological states.

3.VIII. Materials and Methods

Zebrafish Strains and Care

Adult and embryonic zebrafish were raised and cared for using standard laboratory procedures (Westerfield, 2000). I used the following zebrafish mutant and transgenic strains: toh^{s8}, toh^{s220}, toh^{sk12}, toh^{s420}, mil^{m93}, *and* $tg(0.7her5::gfp)^{ne2067}$ (Tallafuss and Bally-Cuif, 2003).

Immunohistochemistry, Fluorescence microscopy and confocal analysis

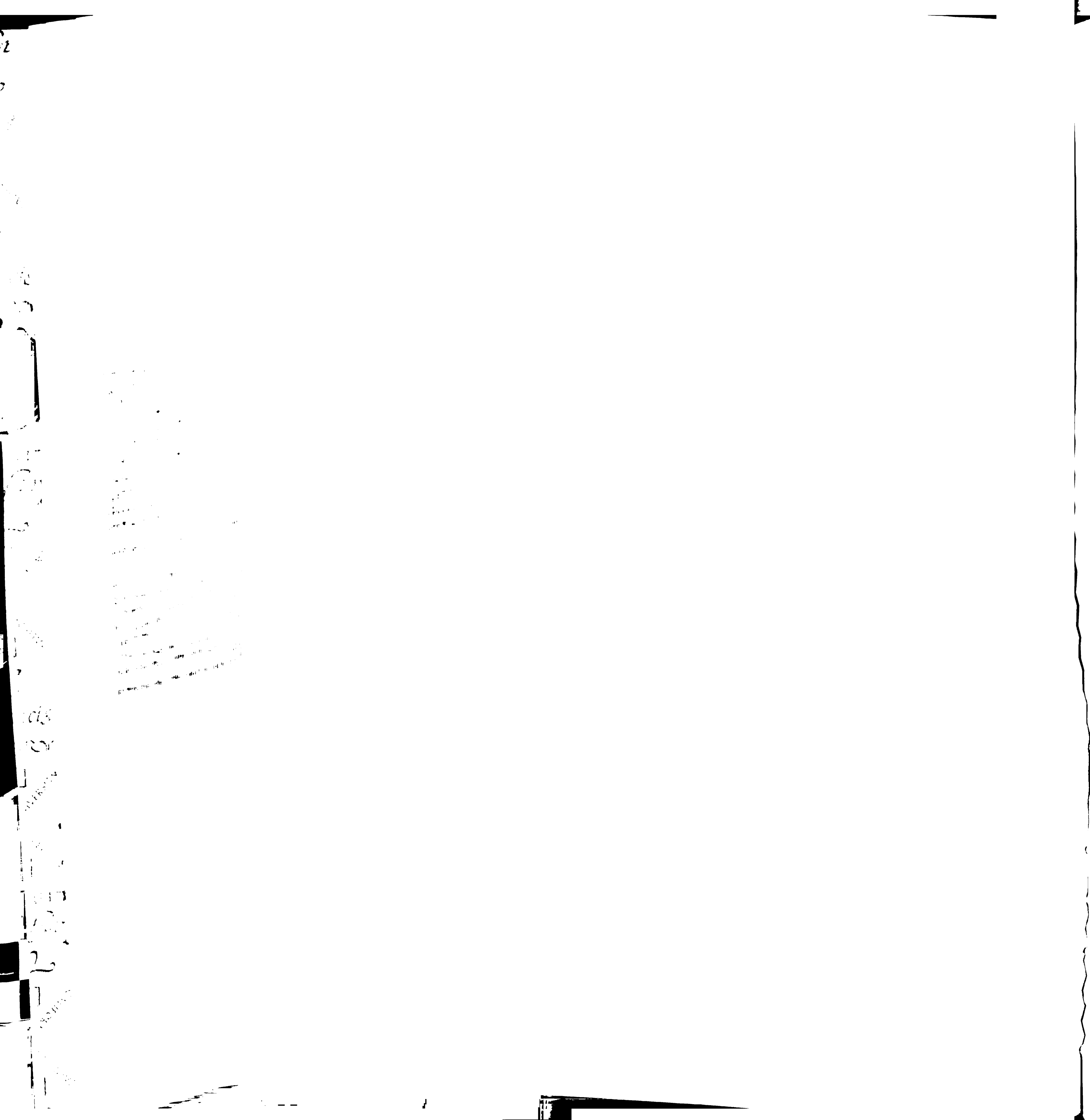
Embryos were fixed at room temperature for 1 hour in 4% Paraformaldehyde in PBS or overnight at 4C in 2% Paraformaldehyde in PBS. The following antibodies were used at the indicated dilutions: mouse monoclonal anti-HA epitope (6E2; Cell Signaling Technologies) at 1:200 (Wilbanks et al., 2002) and rabbit anti-Myc tag epitope (ab9106; Abcam) at 1:200. Actin staining was carried out using Alexa647-phalloidin (Molecular Probes) at 1:50 dilution. All staining was carried out in PBS/1% Dimethyl Sulfoxide/1% Bovine Serum Albumin/ 0.1% Triton X-100. Images were aquired using a Zeiss LSM5 Pascal confocal microscope. Wholemount fluorescence microscopy was performed with a Zeiss SteREO Lumar.V12 microscope.

In situ hybridization

Wholemount in situ hybridization was carried out as described (Alexander et al., 1998) using the following probes: *cmlc2 (Yelon et al., 1999), foxA2 (Odenthal and Nusslein-Volhard, 1998)* and *toh.* The *toh* probe was generated by cloning the full length coding sequence of the *toh* message into pCS2+, linearizing this construct with EcoRI and transcribing with T3 polymerase.

RNA overexpression and morpholino oligonucleotide injections

All RNA generated for injection was generated using mMessage mMachine kits (Ambion). *mil* RNA was generated as described (Kupperman et al., 2000). Embryos were injected with 100 pg of *mil* RNA. pCS2+ *s1p1* was generated by cloning the *s1p1* coding sequence into pCS2+. pCS2+ *milE129A* and pCS2+ *s1p1 E100A* were generated using the QuikChange II Mutagenesis kit (Stratagene) using the primers 5'-ATA CGC GCG GGG ACT GCT TTC ATT GCT TTG TCC GC-3' and 5'- CGC AAT GGT TCT TCA GAG CAG GGA GTA TGT TTG TGG CCT TG-3', respectively. These constructs were linearized with NotI and transcribed using SP6 polymerase. Embryos were injected with 200 pg of *s1p1* or *s1p1 E100A* RNA and 100 pg of *mil E129A* at the one-celled stage. RNA encoding *toh* was generated by linearizing pCS2+ *toh* with NotI and transcribing with SP6 polymerase. Embryos were injected with 100-200 pg of *toh* RNA. A C-terminal Myc epitope tag was added to *toh* with the reverse primver 5'- TTT TCC GCT CGA GTC ACA AGT CCT CCT CGG AGA TCA ATT TCT GCT CTT TGG TAA CCT TAA CTG TTG ACG GTG GCC-3' and cloned into pCS2+. RNA was generated by linearizing pCS2+ *tohcmyc* with NotI and transcribing with SP6 polymerase. Zebrafish



spinl3 RNA was generated by subcloning the coding region of *spinl3* into pCS2+, linearizing this construct with NotI and transcribing with SP6 polymerase. *Drosophila spinster* RNA was generated by subcloning *spinster-RFP* from pUAS *spinster-RFP* (gift of S. Sweeny and G. Davis) into pCS2+, linearizing with NotI and transcribing with the SP6 polymerase. Zebrafish *nrs* was generated from pCS2+ *nrs* (Young et al., 2002). *rab5c-YFP* RNA was generated from pCS2+ Rab5c-YFP (gift of C-P. Heisenberg) as described (Scholpp and Brand, 2004) and embryos were injected with 100 pg of RNA. *HA-mil* RNA was generated by linearizing pcDNA3.1 *HA-miles apart* (gift of Marc Caron) with NsiI and transcribing with T7. Embryos were injected with 100 pg of *HA-mil* RNA.The *toh* morpholino (5'-GCA GCT CTT ACC CTC AGT GCC CAG T-3') was designed by Gene-Tools, Inc. to cover the Exon/Intron boundary between Exon 4 and Intron 4 and prevent splicing of the *toh* message. Embryos were injected with 1 ng of *tohMO* at the one-cell stage of development.

Cloning of the *toh* locus

ankdCAP4 is a marker based on a single nucleotide polymorphism (SNP) in an ORF encoding a zebrafish homologue of the human *ANKHZN* gene. The marker was amplified with the primers 5'- GTT AAC CCT TTT GAA GAC GAG GCT CGA GT-3' and 5'-GAG GAA GAC ATG CCA CGA TT-3' and cut with HinfI to detect the polymorphism. These primers were designed by the dCAPS 2.0 web-based program found at http://helix.wustl.edu/dcaps/dcaps.html (Neff et al., 2002). Zebrafish CA repeat microsatellite primers were obtained from the Massachusetts General Hospital MGH/CVRC Zebrafish Server website (http://zebrafish.mgh.harvard.edu/).

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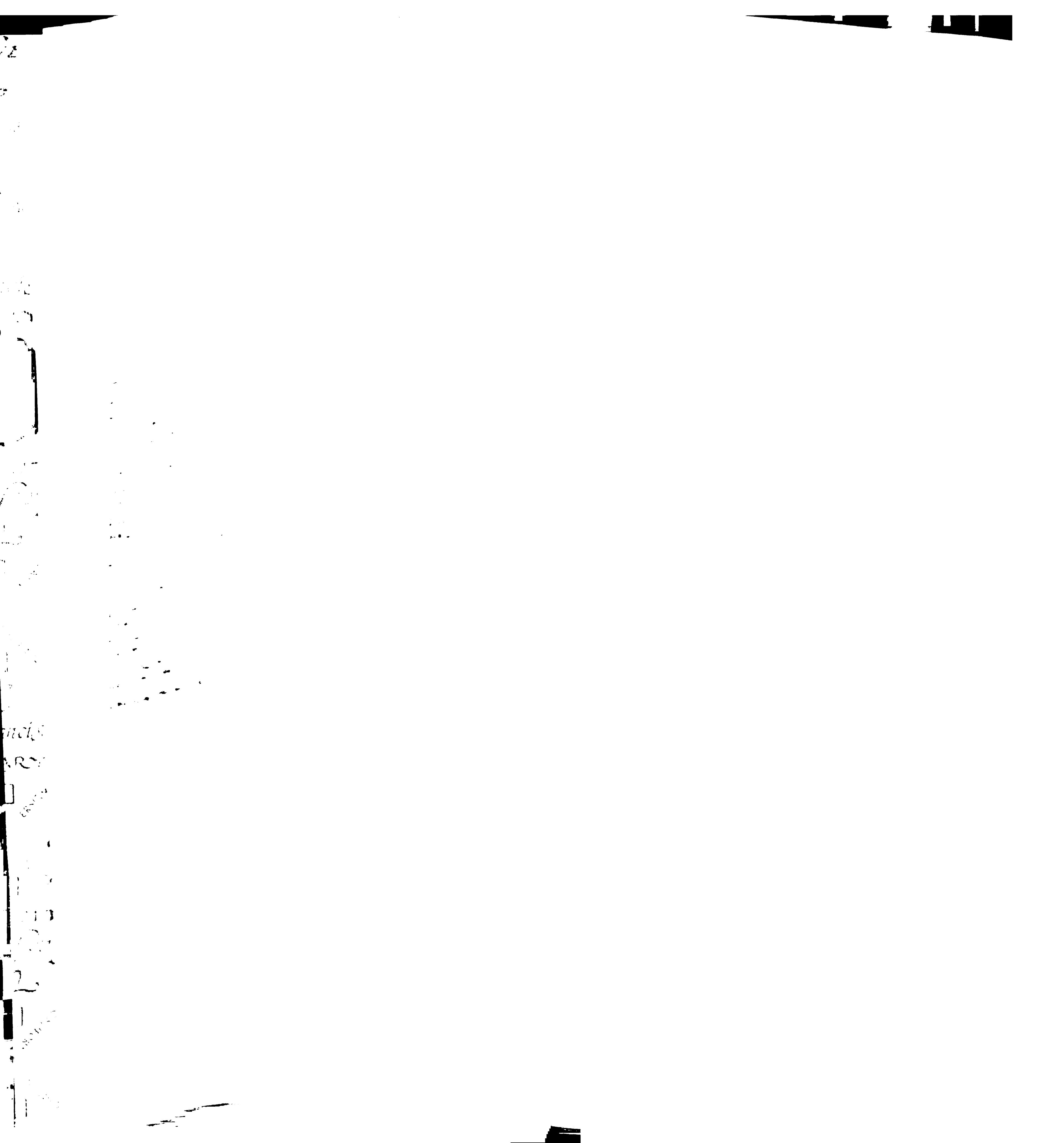

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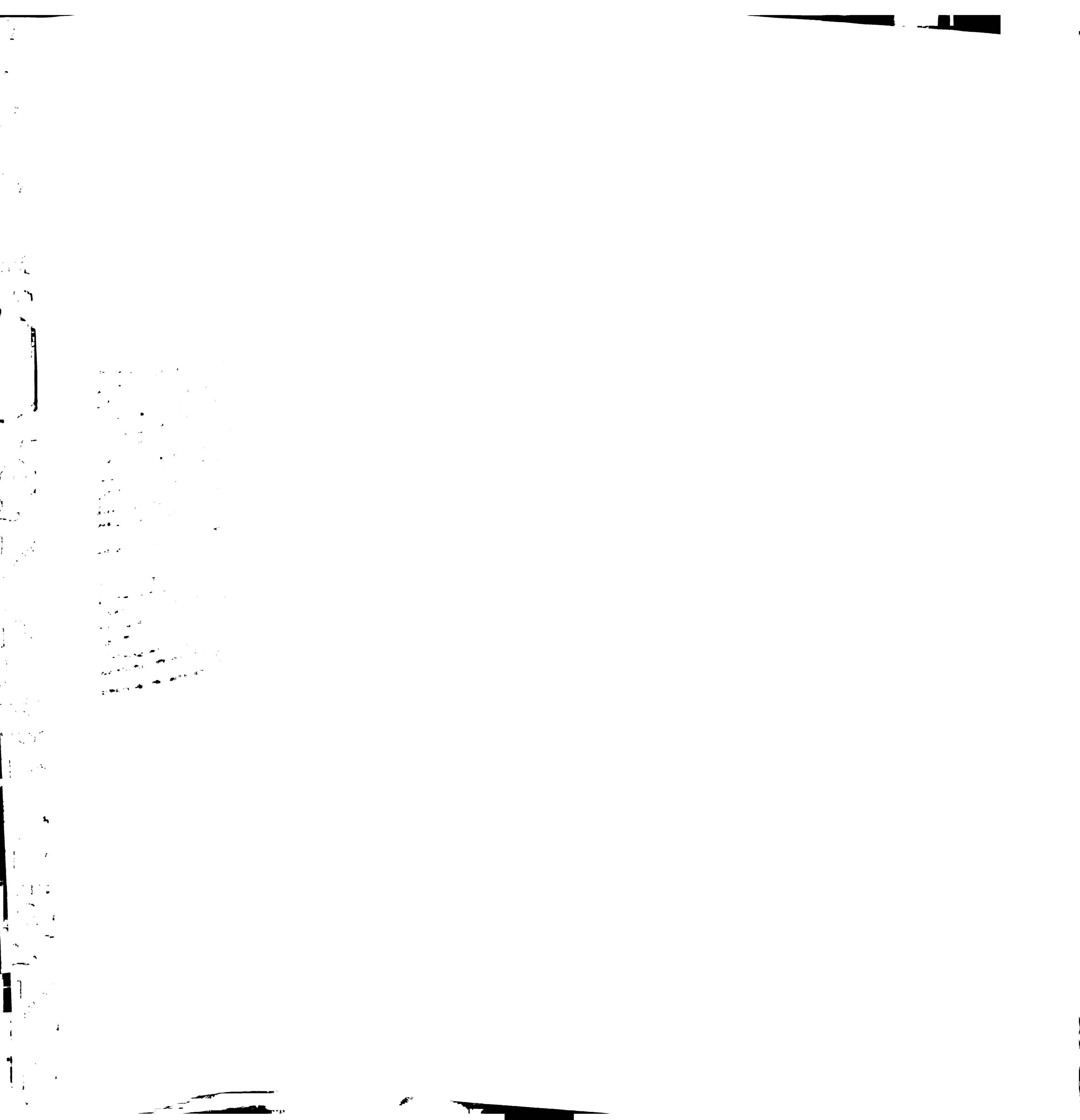

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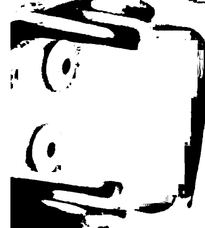
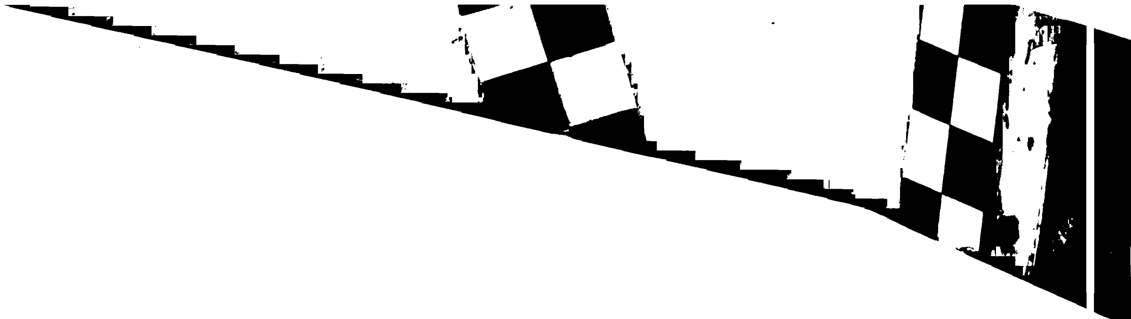

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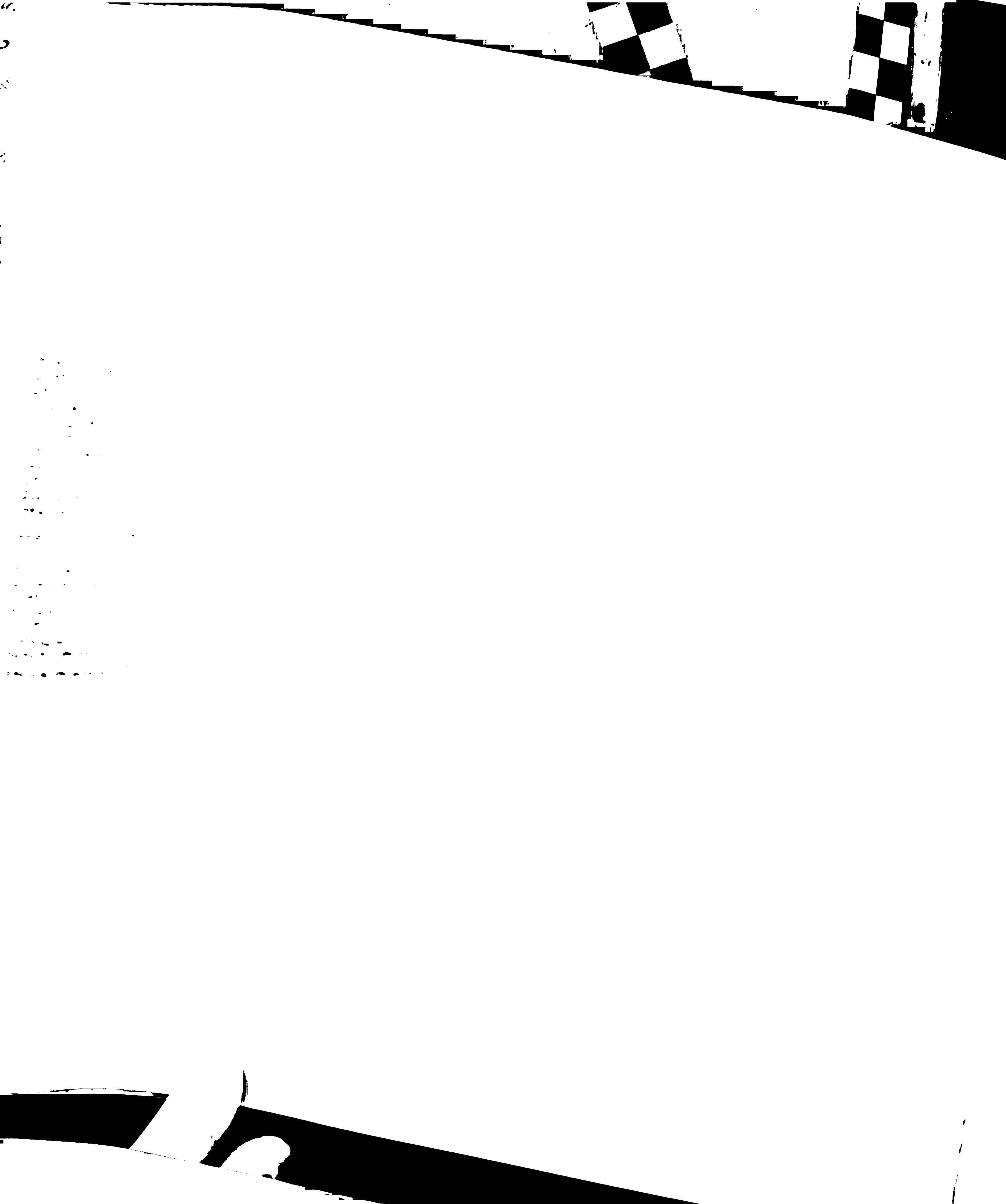

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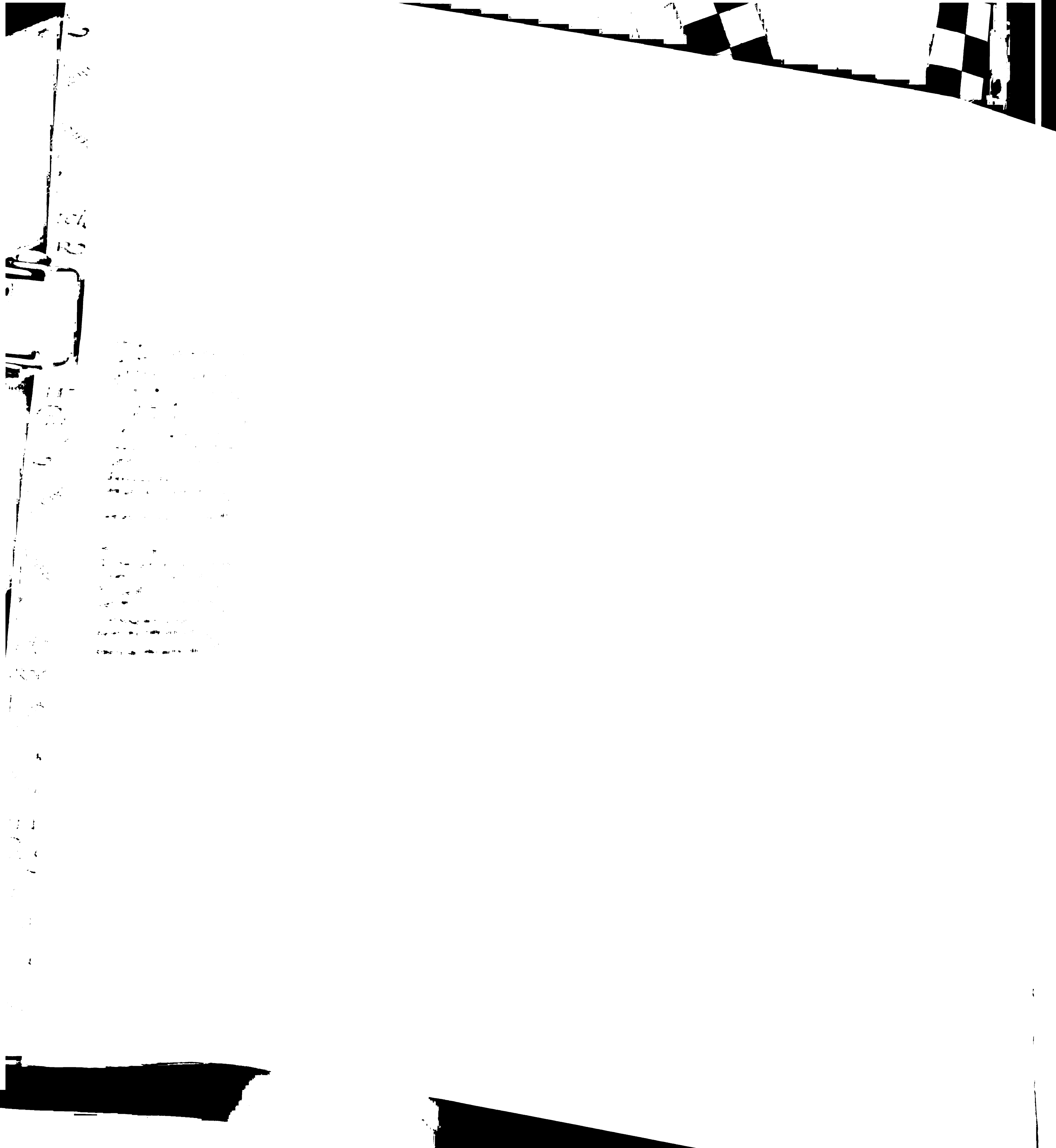

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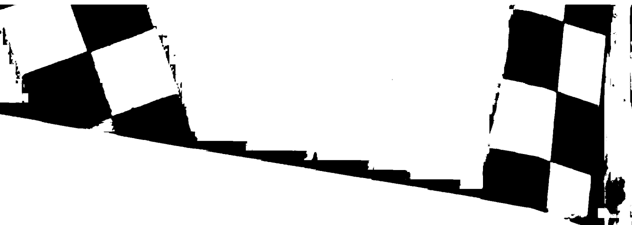

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Appendix A: Overexpression constructs

This appendix contains a table (Table A.1) listing the constructs that I have used to generate RNA for overexpression. The table lists the names of the constructs, whether or not I was able to generate quality RNA from the construct, whether or not overexpression (o/e) caused a phenotype in wild-type embryos, the amount of RNA (in picograms per embryo) injected, whether the overexpression phenotype was affected by *two of hearts* loss of function, and notes about the construct. Of particular interest is the constructs that generated the *m93* allele of *miles apart (mil)* with a 30 amino acid truncation and the constitutively active zebrafish $G\alpha_{12}$ G protein subunit.

The truncated *m93 mil* construct was initially used to test whether or not the *m93* allele is defective in signaling due to constitutive internalization via β-arrestin mediated desensitization. The hypothesis that *m93* might be constitutively desensitized came from the similarity between the *m93* lesion and lesions seen in constitutively desensitized human vasopressin and α_{1B} adrenergic receptors (Wilbanks et al., 2002). *m93* without the truncation fails to cause normal *mil* overexpression phenotypes. However, when the last 30 amino acid residues of *m93*, which contain the hypothetical β-arrestin interaction residues, are deleted *m93* overexpression causes phenotypes similar to overexpression of *mil.* This finding suggests that *m93* is able to signal normally when it lacks the last 30 amino acid residues.

Although the truncated *m93* construct was initially used to determine the nature of the defect in *m93* mediated signaling, it also gave some information about the *two of hearts (toh)* gene. One hypothesis for how *toh* might be functioning in signaling by *mil* is that *toh* is required for the appropriate recycling of Mil back to the plasma membrane

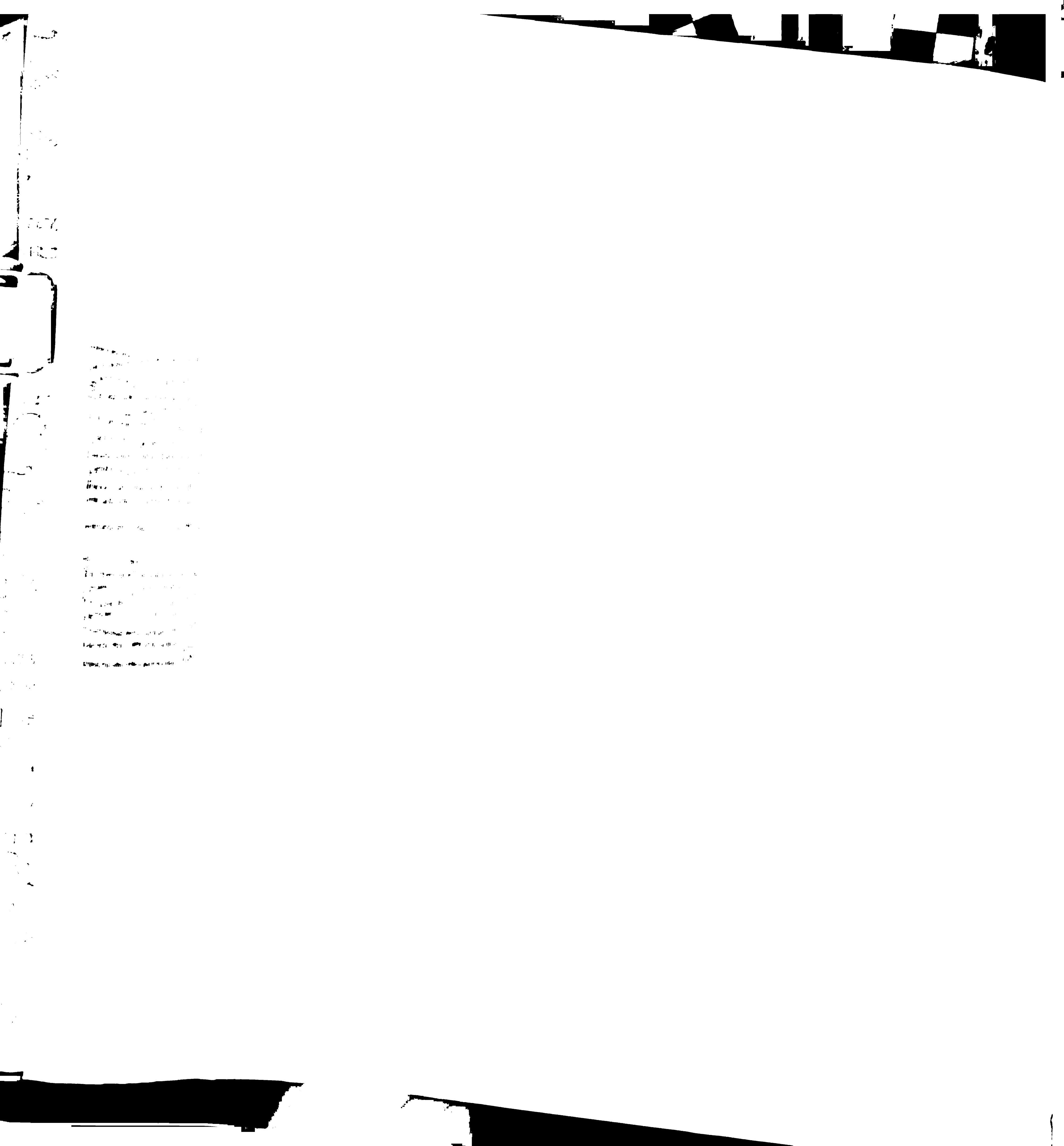

Table A.1: Overexpression constructs

Construct	Quality RNA?	o/e phenotype	Amount injected (pg/embyo)	Rescued by *two of hearts* (*toh*) mutations?	Notes
pcDNA3.1 HAmil	Yes	Yes	100	Yes	HA-epitope tagged *miles apart (mil)*
pCS2+ mil E129A	Yes	No	100 and 200	n/a	Ligand binding mutation; *mil* requires S1P binding for phenotypic effects of overexpression
pCS2+ milΔ30	No	?	n/a	?	30 aa truncation; should block β-Arrestin binding and internalization
pCS2+ HAmilΔ30	No	?	n/a	?	30 aa truncation with HA-epitope tag, unable to verify sequence
pCS2+ m93Δ30	Yes	Yes	50 and 100	Yes¥	30 aa truncation of *m93* allele, should block β-Arrestin binding and internalization
pCS2+ m93Δ30 E129A	Yes	No	100 and 200	n/a	Ligand binding mutation in *m93* allele of *mil* with 30 aa truncation
pCS2+ mil YR	Yes	Yes	100	Yes	Zebrafish *mil* with human $S1P_2$ ligand binding domain; testing relative S1P affinities for human and zebrafish $S1P_2$
pCS2+ mil NR	Yes	Yes	100	Yes	Zebrafish *mil* with partial human $S1P_2$ ligand binding domain; partial zebrafish ligand binding domain remains
pCS2+ HAs1p1	Yes	Yes	100, 150 and 200	No	$S1P_1$ orthologue, not rescued by *toh* mutations
pCS2+ HAs1p1 E100A	Yes	non-specific	100 and 200	n/a	Ligand binding mutation in $S1P_1$, shows non-S1P dependent phenotypes
pCS2+ s1p3	Yes	Yes‡	100, 150 and 200	No	$S1P_3$ orthologue; not apparently rescued by *toh* mutations
pCS2+ Galpha12 QL	Yes	Yes	25 and 50	partial†	Constitutively active zebrafish $G\alpha_{12}$; disrupts epiboly movements and convergent extension
pCS2+ Galpha 13A	No	?	n/a	?	Wild-type zebrafish $G\alpha_{13A}$
pCS2+ Galpha 13A QL	Yes?	Weak	50 and 100	?	Constitutively active zebrafish $G\alpha_{13A}$; no phenotype; RNA quality?
pcDNA3.1 hGalphi QL	No	?	n/a	?	Constitutively active Human $G\alpha_{i2}$
pcDNA3.1 hGalphaq QL	No	?	n/a	?	Constitutively active human $G\alpha_q$
pCS2+ toh	Yes	No	100 and 200	Rescues *toh* mutations	Wild-type *toh*
pCS2+ tohcmyc	Yes	No	25, 50 and 200	Rescues *toh* mutations	C-terminal myc-epitope tagged *toh*
pCS2+ APJ (*grinch*)	Yes	Yes	100	No?	Apelin receptor; single injection experiment

n/a = not applicable

¥ *toh* is unlikely to function Mil recycling; this construct would be predicted to generate a Mil that is not internalized or de-sensitized and *toh* mutations still rescue its overexpression. The hypothetical internalization defects of this construct have not been verified, however.

‡ *s1p3* overexpression causes low penetrance shortened or disrupted body axis defects. These effects may be dependent on S1P interaction with $S1P_3$ or may be ligand independent.

† See Figure A.1 for the quantitation of "partial rescue"

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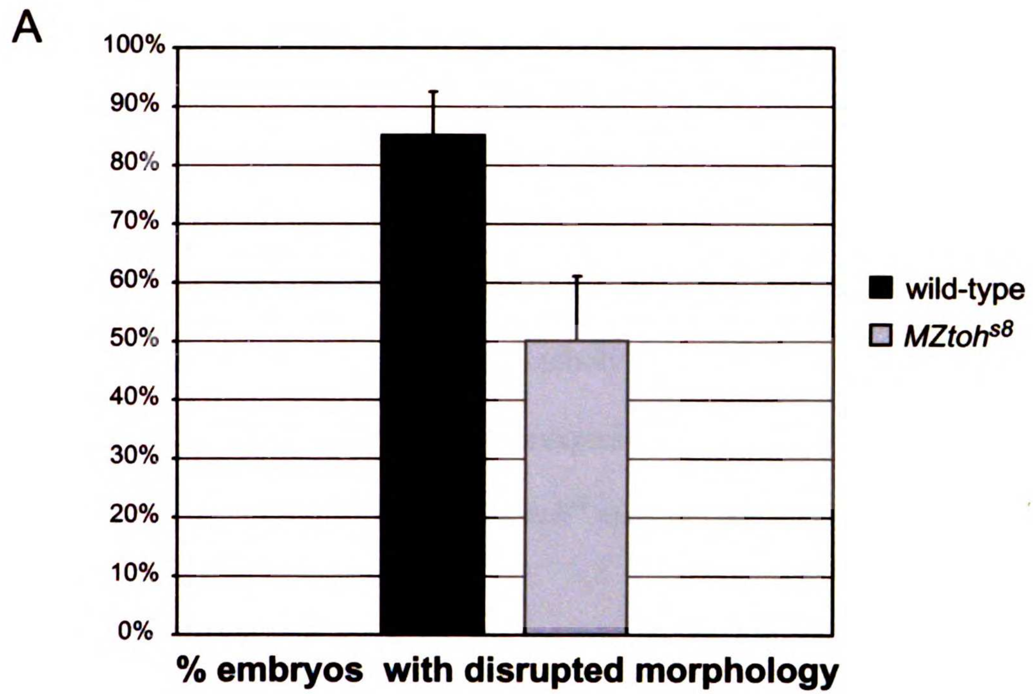
after it is internalized due to signaling mediated desensitization. However, the *toh* mutation rescues overexpression of *m93Δ30* RNA. This result suggests that *toh* mutations are still able to rescue *mil* overexpression phenotypes even when the receptor is unlikely to be efficiently internalized. There is a caveat to this interpretation, however, as the internalization defects of this construct have not been verified.

Finally the constitutively active zebrafish $G\alpha_{12}$ construct has given further suggestions as to how *toh* might function. This construct is the zebrafish $G\alpha_{12}$ G protein subunit engineered with a mutation changing the glutamine at position 235 mutated to a leucine. This type of mutation is known to confer constitutive signaling properties to G protein alpha subunits (Graziano and Gilman, 1989). Interestingly the overexpression of constitutively active $G\alpha_{12}$ is partially rescued by *toh* mutations (Figure A.1). The defects caused by overexpression of constitutively active $G\alpha_{12}$ include failure to complete epiboly movements, shortened body axis and head defects such as cyclopia. However, the rescue of $G\alpha_{12}$ Q235L overexpression by *toh* mutations is not as clear as with overexpression of *mil,* as many of the *toh* mutant embryos still have defects in their morphology when overexpressing $G\alpha_{12}$ Q235L RNA. This finding might suggest that *toh* is able to affect some level of $G\alpha_{12}$ mediated signaling. Unfortunately these overexpression phenotypes are somewhat difficult to interpret. Because I have expressed a potent signaling molecule at very high levels it is unclear whether these overexpression conditions are mirroring normal $G\alpha_{12}$ signaling. Furthermore, mutations in *toh* may be affecting $G\alpha_{12}$ signaling non-specifically by disrupting a cellular process that $G\alpha_{12}$ requires for signaling. As of yet, no other G protein alpha subunits have given repeatable or scoreable phenotypes from overexpression.

Figure A.1



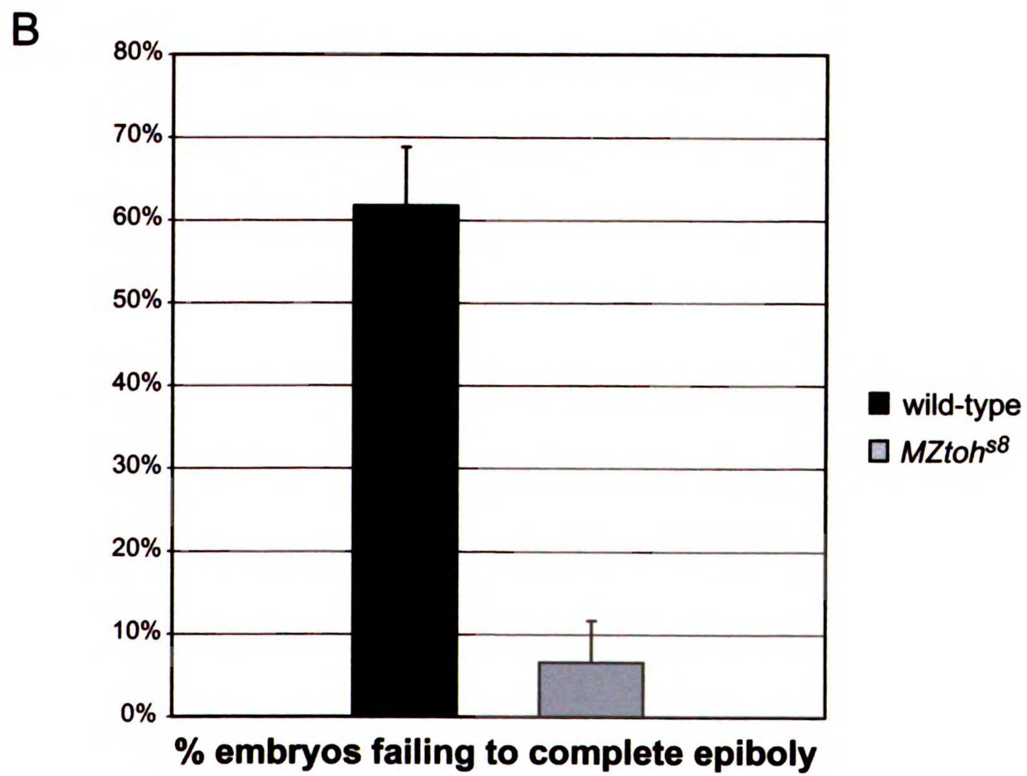


Figure A.1: Overexpression of constitutively active zebrafish Gα_{12}

(A) Percentage of embryos injected with 50 pg of zebrafish *Gα_{12} Q235L* RNA with severe morphological defects, including embryos that fail to complete epiboly. 85.0% (S.D. ± 7.5%, n = 307 embryos) of wild-type embryos (black) have severe morphological defects. Only 50.1% (S.D. ± 11.0%, n = 443 embryos) of *MZtoh^{s8}* have severe morphological defects when overexpression Gα_{12} Q235L. (B) Percentage of the embryos injected *Gα_{12} Q235L* RNA failing to complete epiboly due to epiboly movement defects. 61.7% (S.D. ± 7.1%) of wild-type embryos overexpressing Gα_{12} Q235L fail to complete epiboly movements. 6.6% (S.D. ± 5.0%) of *MZtoh^{s8}* embryos fail to complete epiboly when overexpressing Gα_{12} Q235L.

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The "humanized" *mil* constructs were generated because when compared to other S1P receptors *mil* appears to be missing two conserved residues thought to be required for interaction with S1P (Parrill et al., 2000). These residues are generally a tyrosine or phenalalanine followed by a lysine or arginine. However, in *mil* these residues are two asparagines (N288 and N289). In order to test whether Mil is rescued by *toh* mutations because of a lower affinity for S1P, I generated two mutant versions of the *mil* construct. The first construct, pCS2+ mil YR, has two mutations: N288Y and N289R. This construct is completely "humanized" at these residues in that it matches the human $S1P_2$ sequences. In addition a partially "humanized" construct, pCS2+ mil NR, was generated that only has the N289R mutation. Overexpression of these constructs showed no difference from overexpression of wild-type *mil* in wild-type embryos, suggesting that these constructs are still active. However, *toh* mutations still rescue overexpression of these constructs. This finding indicates that the rescue of *mil* overexpression by *toh* mutations is not dependent on the changes seen at these residues.

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Appendix B: Measurement and manipulation of sphingosine 1-phosphate

B.I. Measurement of sphingosine 1-phosphate in wild-type and *two of hearts* mutant embryos

Because the question of whether *two of hearts (toh)* gene function might be required for the production or release of sphingosine 1-phosphate (S1P) arose, I wanted to measure the amount of S1P present in wild-type and *toh* mutant embryos. To do this I collected pools of wild-type and maternal zygotic *s8* ($MZtoh^{s8}$) mutant embryos. Each pool consisted of twenty embryos at 70% epiboly or 7.5 hours post fertilization (hpf). These embryos were suspended in 500 µL 10% Hanks' Basic Salt Solution /1 mM HEPES buffer and vortexed at low speed briefly to dissociate the embryos. The embryos were then spun down gently (300 rpm in an Eppendorf microfuge) for 1 minute. The embryos were resuspended in 150 µL RPMI + fatty acid free bovine serum albumin (FAF-BSA) media (described in Schwab et al., 2005). The dissociated embryos were vortexed briefly and incubated in the RPMI/FAF-BSA media for 2 minutes. The pools were then spun down at low speed (see above) for 1 minute and the supernatant was collect with care not to disturb the pellet.

The supernatants for eight wild-type and five $MZtoh^{s8}$ mutant embryo pools were analyzed for the presence of S1P using an established bioassay (Schwab et al., 2005) (Figure B.1A). Briefly, serial dilutions were made of each embryo pool supernatant. These dilutions were added to WEHI231 cells stably expressing FLAG-S1P$_1$ and incubated at 37C. The cells were then incubated with fluorescently labeled anti-FLAG

antibody to mark cell surface FLAG-S1P_1 and then FACs sorted. The mean fluorescent index (MFI) for each pool was then measured. Because cells that have been exposed to S1P rapidly internalize their FLAG-S1P_1 in an S1P dose dependent fashion, cells exposed to higher S1P concentrations should have lower MFIs.

Using this assay there is no detectable difference between wild-type and *MZtoh*^{s8} pools with respect to their MFIs (Figure B.1A), suggesting that there is no difference in S1P levels in the pools. Unfortunately, because of the way the supernatants were generated (dissociation of whole embryos) it is difficult to say what pool of S1P is being measured in this experiment. Although I attempted to dissociate the embryos as gently as possible to maintain cellular integrity, these measurements may, in fact, be the entire pool of intracellular and extracellular S1P in these embryos instead of merely the S1P being released by intact cells. In addition, the yolk cells of the dissociated embryos may contain maternally deposited S1P that might contaminate the supernatants. Therefore, all that can be determined from this experiment is that S1P is present in wild-type and *toh* mutant embryos at this stage of development.

One method that might be employed to measure only the extracellular S1P contribution of wild-type or *toh* mutant cells would be to use cell culture. Dissociated embryo cell culture would perhaps be the best method. I have had some success growing cells from dissociated embryos for several days at 28C on poly-L-lysine coated dishes in amphibian L15 media with 15-20% fetal bovine serum. After two days, however, the cells tend to differentiated into heterogeneous cell populations. Mechanically or enzymatically dissociated wild-type and *MZtoh*^{s8} cells could be washed multiple times to remove all contribution of the yolk or disrupted cells to the S1P measurements. Once the

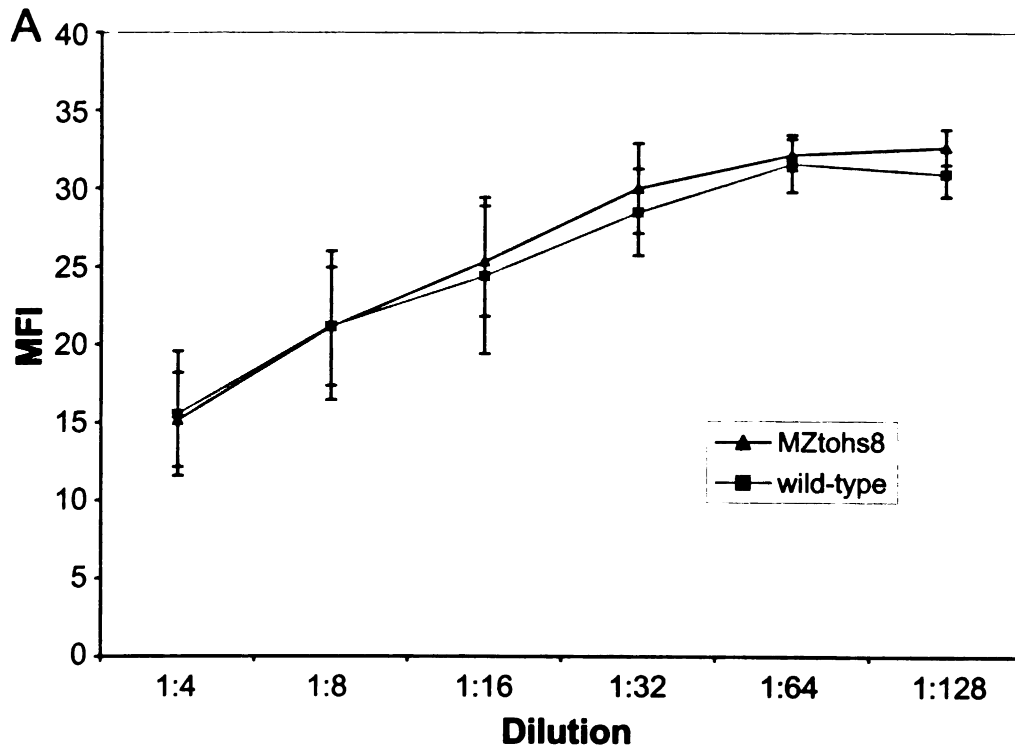
intact cells had adhered to plates, one could add RPMI/FAF-BSA media to the dishes and incubate to collect S1P for measurements. The media could then be pipetted off without disrupting the cells. The benefit of this method is that the only S1P picked up in the media would be either released by or present in the outer leaflet of the plasma membrane of cells. Furthermore, the cells could be grown on the plates for several hours before adding the collecting media allowing them to recover from the trauma of dissociation and centrifugation. Therefore, the S1P measurements would be more likely to be closer to the in vivo behavior of the cells.

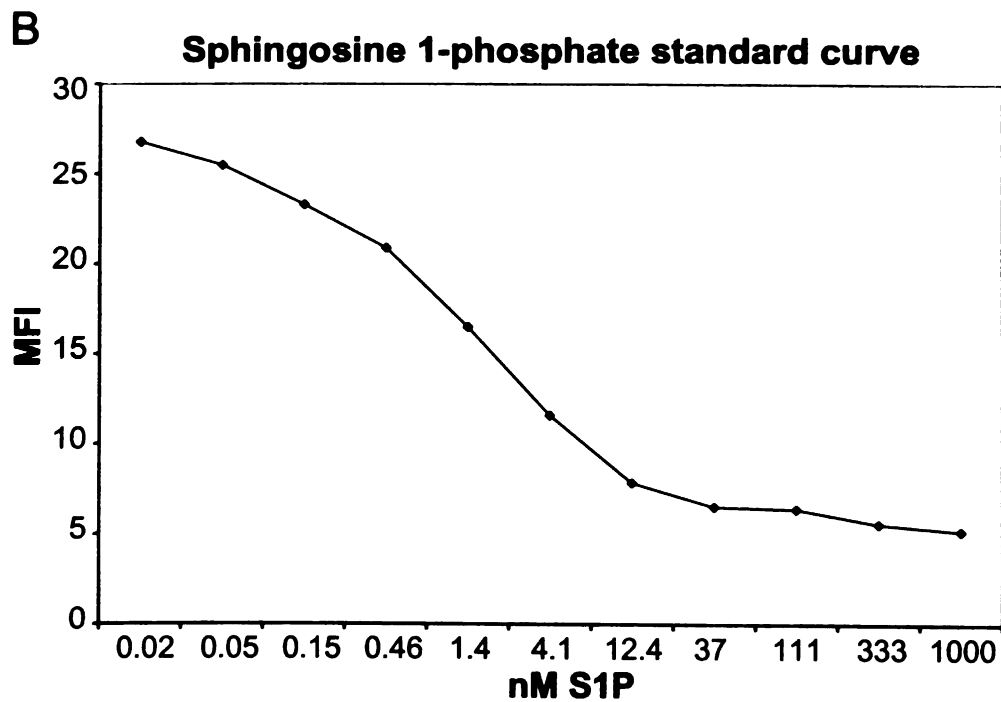
An S1P standard curve (Figure B.1B) is included for comparison between the embryo supernatants and known S1P concentrations. This curve suggests that the S1P concentrations of the supernatants are an estimated 3.5 to 5.5 nM. It should be noted that this S1P concentration in the supernatants collected from the embryos is based on the arbitrary amount of media I added to the pelleted cells. Therefore, this concentration of S1P is not indicative of actual concentrations of S1P in the embryo.

B.II. Manipulation of sphingosine 1-phosphate

I have attempted several methods for manipulating the levels of S1P signaling in embryos. First, I have attempted to reduce the S1P signaling in embryos by expressing two constructs that would be expected to disrupt S1P production or to lead to increased S1P degradation. The RNAs from constructs encoding a dominant negative (DN) mouse sphingosine kinase (SK) 1 and human S1P lyase have been injected into wild-type embryos (100-200 pg RNA/embryo). Neither of these constructs had any effect on the development of the embryos. It had been hypothesized that either of these constructs

Figure B.1





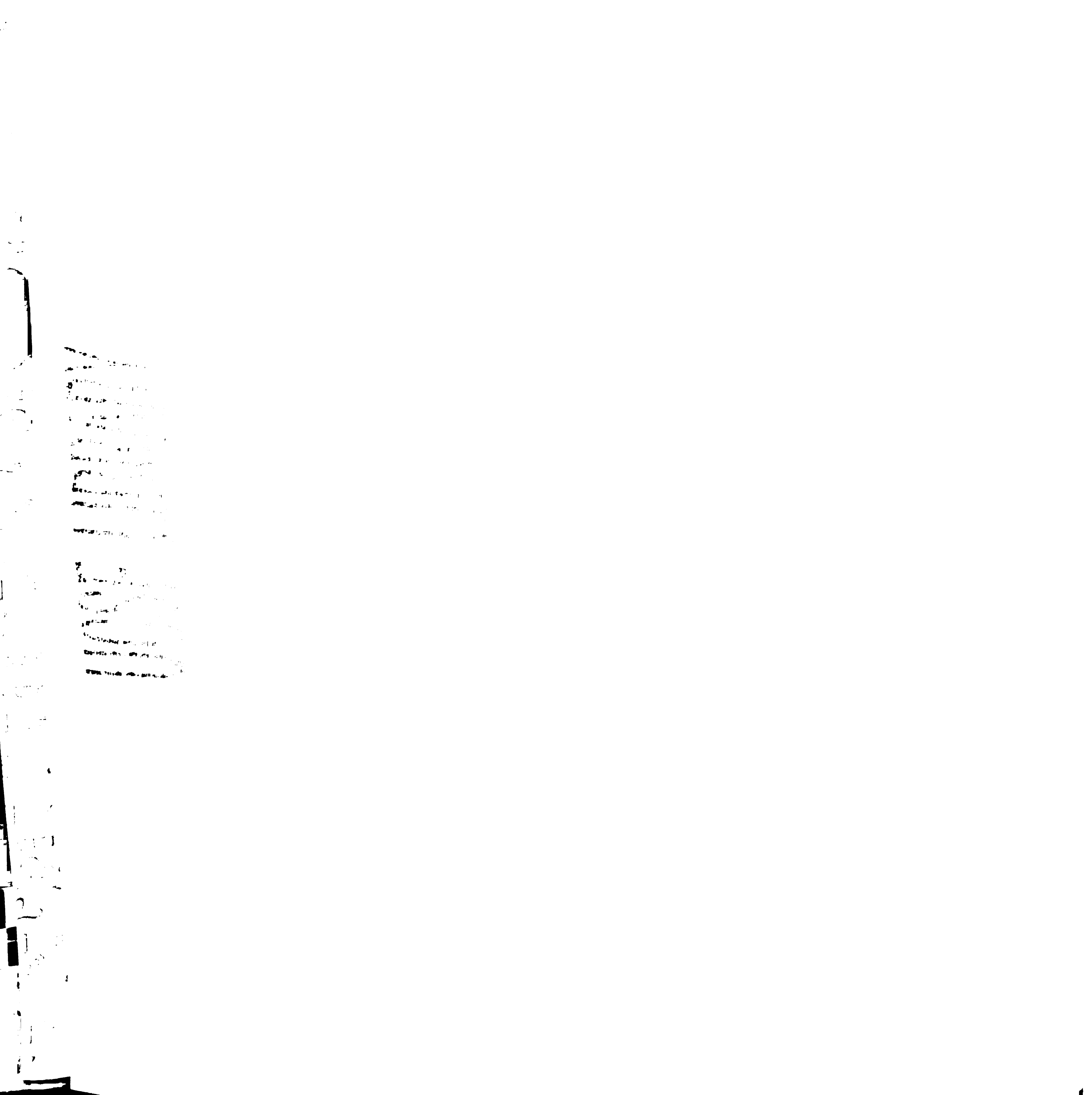
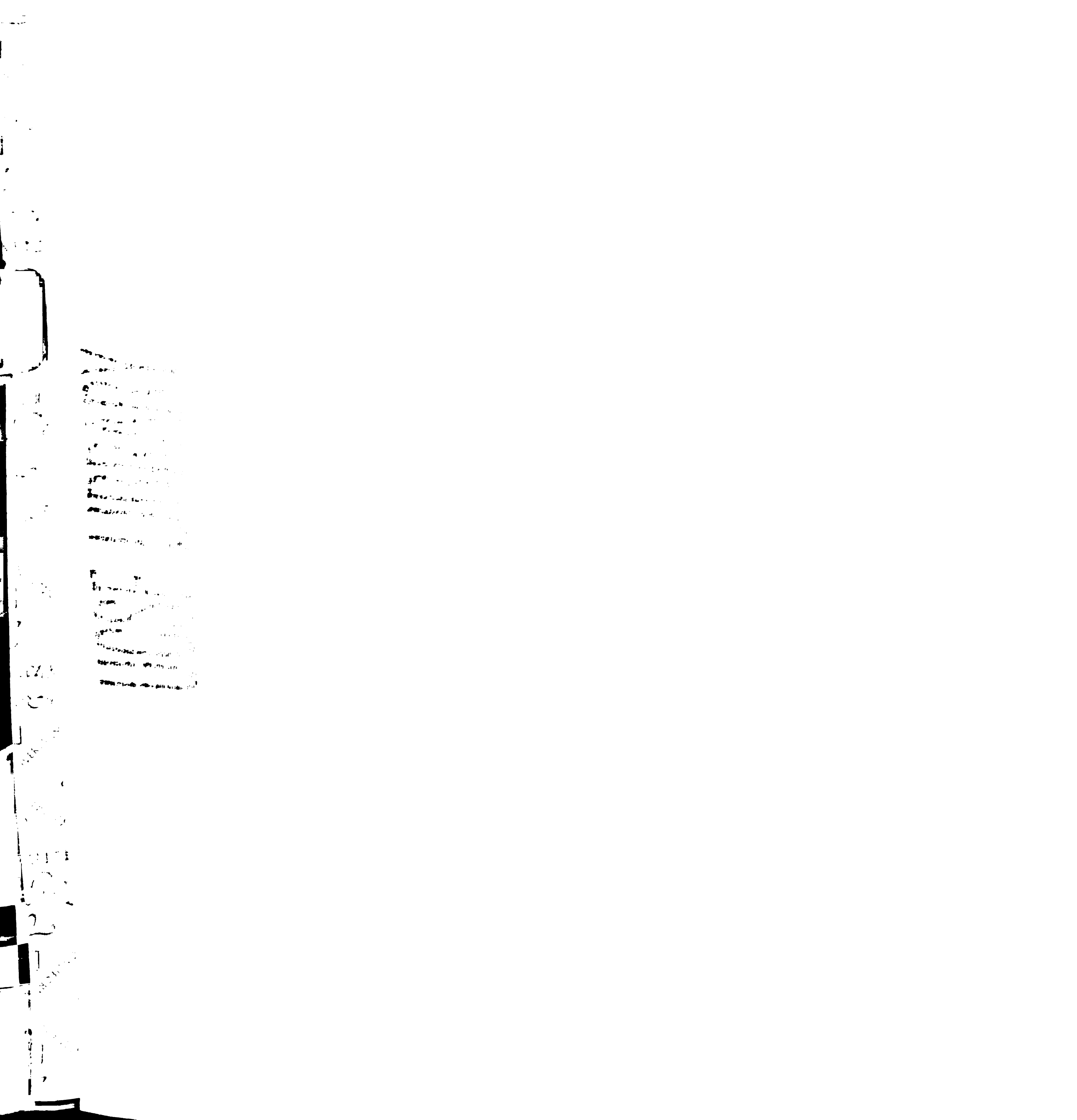


Figure B.1: Measurement of sphingosine 1-phosphate in wild-type and *two of hearts* mutant embryos.

(A) Comparison of levels of S1P from wild-type (red, squares) and *MZtoh*^{s8} (green, triangles) embryo extracts. Mean fluorescent index (MFI) was measured for FLAG-S1P$_1$ expressing WEHI231 cells exposed to the indicated dilution of each pool then incubated with fluorescently tagged anti-FLAG antibody to examine surface FLAG antigen. For each dilution data point the average of eight wild-type or five *MZtoh*^{s8} embryo pools. Error bars indicate standard deviation amonst the pools at each dilution. (B) Standard curve using known S1P concentrations. Known S1P concentrations were added to the FLAG-S1P$_1$ expressing WEHI231 and these cells were treated as the cells incubated with the embryo extract pools.



might lead to impaired S1P signaling and might phenocopy the ***miles apart (mil)*** mutant. However, this was not the case. One caveat is that the human S1P lyase construct was not cloned into a vector containing a poly-adenylation signal and, therefore, the RNA from this construct might be fairly unstable after injection. The mouse DN-SK1 was cloned in pCS2+ and should contain the SV40 poly-adenylation signal.

In addition to attempts to negatively affect S1P signaling in embryos, I have also attempted to increase the available S1P or to enhance S1P signaling. To increase the available S1P in embryos I injected wild-type, *toh* and *mil* mutant embryos with wild-type mouse SK1 (100 pg RNA/embryo). Overexpression of SK1 had no detectable effect on wild-type, *toh* or *mil* mutant embryos. In addition to SK1 injections, I attempted to enhance S1P signaling using the S1P receptor agonist FTY720. However, FTY720 also had no effect on zebrafish development, either when embryos were incubated in egg water containing the drug or when embryos were directly injected with 2.3 nL of 10 mM FTY720 each. It should be noted that FTY720 is not expected to affect Mil as this drug is ineffective at activating mammalian $S1P_2$ subtype receptors (Brinkmann et al., 2002; Mandala et al., 2002). Furthermore, it is unclear what the bioavailability of FTY720 in the embryos might be like. The drug may be unable to penetrate embryos to interact with receptors.

Therefore, the attempts to modulate S1P levels or signaling have not been successful at generating developmental phenotypes. However, I have not attempted overexpression of the zebrafish versions of any of the S1P biosynthetic enzymes. The zebrafish genes might be more successful at functioning in zebrafish embryos.

Furthermore, I have not injected RNA encoding any of the S1P phosphatases that might be successful at degrading S1P in the embryo.

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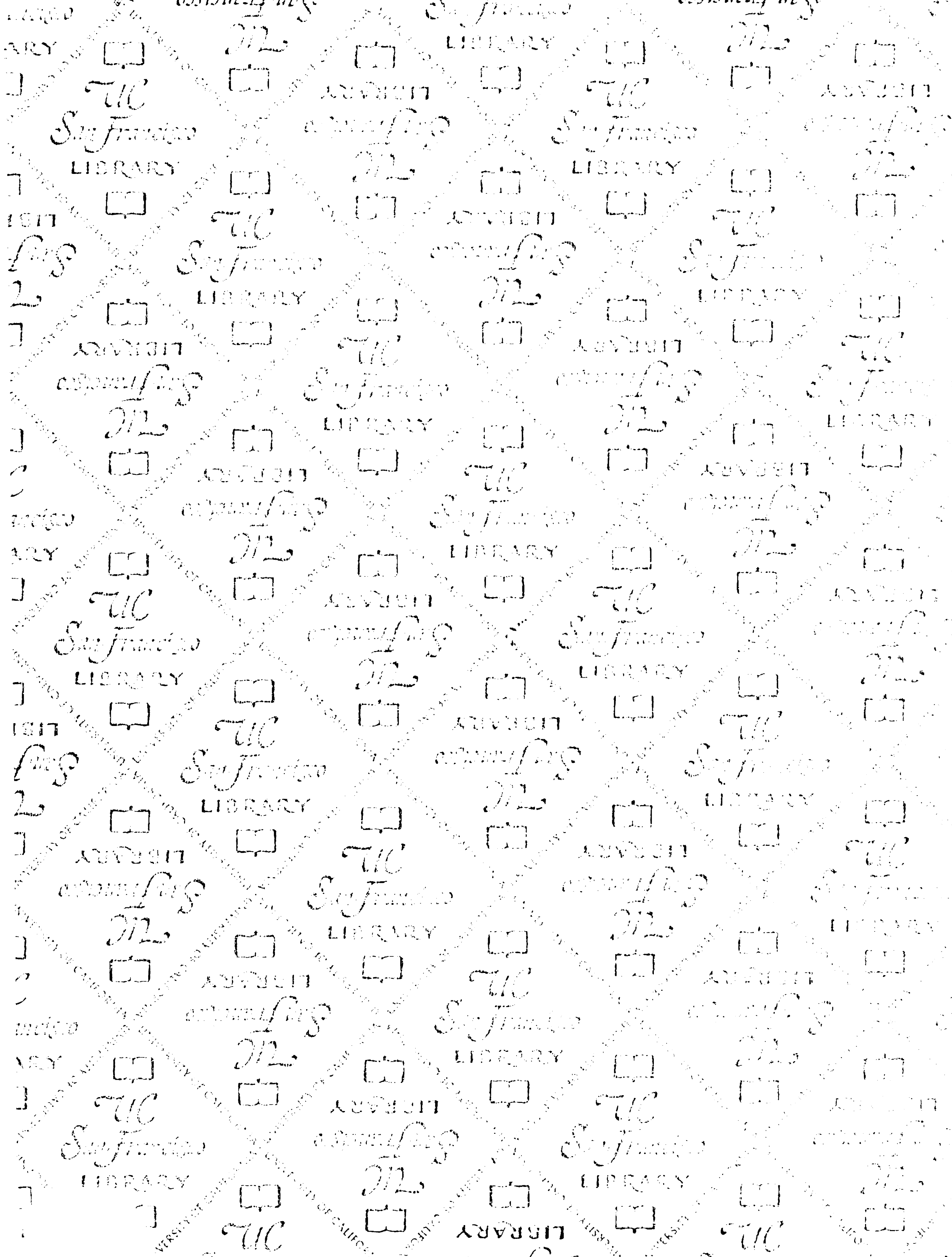

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