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Elucidating the Mechanism of Action of Lmo4 in Vertebrate Eye Development

by

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ABSTRACT

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We have identified the lmo4 locus as a regulator of the size and organization of the eye. Using the yeast 2 hybrid system, we have identified and characterized Lmo4-Ldb interactions. We show that Lmo4 interacts with zebrafish Ldb1, Ldb2, and Ldb3 (LIM-domain binding) proteins, albeit more strongly with Ldb1. This interaction can be mediated by both LIM domains of Lmo4, though the interaction is much stronger with LIM B rather than LIM A. Mutating both LIM domains through strategic point mutations of key zinc coordinating residues abolishes their ability to interact with Ldbs. Mutant overexpression studies showed that overexpressing LIM B results in a mild small eye phenotype whereas overexpressing LIM A does not. Furthermore, Ldb1, Ldb2, and Ldb3 can heterodimerize and all can interact with zebrafish Islet3, which belongs to the LIM-homeodomain class of proteins and is suggested by others to play an essential role in eye development. We have tested the model of islet3 antagonism by lmo4 through morpholino knock down of islet3 and attempted rescue of the lmo4 gain of function phenotype by islet3 overexpression. Knock down of islet3 does not result in an eye phenotype, and rescue experiments show that the lmo4 overexpression phenotype can not be rescued by ectopic isl3 overexpression. We conclude that islet3 is not necessary for eye development in zebrafish, and the role of lmo4 with respect to eye development must be mediated through an antagonism of other lhx genes or through an lhx-independent mechanism.
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INTRODUCTION

Lmo4 belongs to the LIM-only class of proteins. This class consists almost entirely of 2 tandem LIM domains, which are known to mediate protein-protein interactions (Dawid et al., 1998). Thus, proteins containing LIM domains are thought to act as adaptor molecules mediating the formation of protein complexes and modifying the activity of proteins within those complexes. The literature suggests that one prominent mechanism of action of Lmo members is through the antagonism of another class of LIM domain-containing proteins termed the LIM homeodomain or Lhx proteins through competitive binding and sequestering of their essential LIM domain-binding (Ldb) cofactors.

Functional characterization of lmo4 in zebrafish has revealed its role in regulating limb development, craniofacial development, otic vesicle formation, and body pigmentation, as well as the size and organization of the eye during embryogenesis (McCollum et al., submitted and preliminary results). We have used zebrafish eye development as a bioassay for studying the mechanism of action of Lmo4. Specifically, we have sought to elucidate the cellular and molecular functions of Lmo4 during eye development by identifying and characterizing Lmo4-Ldb interactions and ascertaining the
functional significance of those interactions in the embryo. This work will add to the understanding of how Lmo members and other LIM domain-containing function during development.

SIGNIFICANCE

Living organisms receive environmental stimuli through their sensory organs. The eye is the organ of sight, which is arguably our most precious sense. This work will help to elucidate the underlying mechanism of normal development and different birth defects affecting eye development such as anophthalmia and microphthalmia, medical terms used to describe the absence or reduction of the globe and ocular tissue from the orbit, and coloboma, which is a congenital eye defect in which part of the eye structure is missing causing eventual blindness. We believe, for reasons that will become clear throughout this paper, that lmo4 is involved in regulating the specification and patterning of the different cell types in the optic primordia, so this project will give us a better understanding of general cell type specification events which are essential for normal embryonic development. Additionally, our preliminary functional analysis of lmo4 has implicated it in limb, craniofacial, ear, and pigmentation defects as well. Thus, studying it’s
mechanism of action in regulating cell-type specification in the eyes will elucidate its role in a host of other developmental processes. Furthermore, misexpression of members of the \textit{lmo} gene family are known to be associated with childhood leukemia (Rabbitts, 1998) and breast cancer (Rabbitts, 1998; Visvader et al., 2001). Therefore, an analysis of their role in normal development will lead to a greater understanding of their oncogenic mechanism.

\textbf{BACKGROUND}

This background section presents a broad overview of the current knowledge pertaining to this thesis. We start with a description of the isolation and characterization of the \textit{lmo4} locus. Expression studies and functional studies implicating a role in various developmental processes including eye development in described. \textit{Lmo4} belongs to the LIM only class of proteins. We present current knowledge of the different classes of LIM domain-containing proteins and their mechanisms of action through protein-protein interactions. Studies suggest Lmo proteins exert their function through two primary mechanisms: Lhx antagonism and the
mediation of multimeric protein complexes. Then, we describe candidate interactors to Lmo4 and candidate targets for antagonism by Lmo4. We then explain the advantages of using zebrafish as a vertebrate model organism, particularly with respect to eye development. We describe the process of embryogenesis in zebrafish and illustrate the development process of early eye formation.

**Characterization of lmo4**

*Lmo4* was cloned in a subtractive hybridization cDNA screen for genes expressed in the neural plate during gastrulation (Sagerstrom et al., 2001). Its expression was found to be dynamic throughout the early embryo, particularly in the developing eyes (Lane et al., 2002). During neural plate stages, it’s expressed in the presumptive telencephalon, rostral and lateral to the eye field, as well as diffusely in the mesoderm and tailbud (Figure 1A-B). As neurulation takes place, expression persists in the dorsoanterior neural keel, head mesoderm, somitic and presomitic mesoderm, and tailbud (Figure 1C-D). At 10-somite stage, it is expressed in much of the optic vesicles and telencephalon, although the expression becomes asymmetrical with stronger staining in medial regions of the optic vesicles. As development progresses, the expression becomes more and more restricted
until only the presumptive optic stalk and presumptive RPE show expression (Figure 1E-H).

![Figure 1. Lmo4 expression in the eye. Whole mount in situ hybridization with an lmo4 probe. A, C lateral views. B, D,E-H. dorsal views. A, B. 3-somite stage. C, D. 6-somite stage. E. 10-somite stage. F. 14-somite stage. G. 18-somite stage. H. 21-somite stage. Reprinted from Lane et al., 2002.](image)

Overexpression of lmo4 results in a variable small-eye phenotype (Figure 2). In the most severe circumstances, the retina is lacking altogether. However, pax2.1 expression (marker for the optic stalk) can be evidenced, which suggests that the optic primordia has been specified to some degree,
and that *lmo4* might play a role in the specification of different cell types within the optic primordia. In addition to a reduced retina, an increase in the number of cells in the RPE is seen (Figure 2). These experiments suggest that *lmo4* blocks retinal specification and induces RPE specification.

**Figure 2. Lmo4 overexpression results in a small eye phenotype.**
A. Embryo injected with 500-750 pg of GFP mRNA at the 1-2 cell stage. B. Embryo injected with 500-75- pg of *lmo4* mRNA at 1-2 cell stage. All embryos are lateral views of 24 hpf embryos stained with a probe for *nor/pax2.1*. White block arrows in B point to ectopic melanophores. Reprinted with permission from Dr. Mary Ellen Lane.

Knocking down *lmo4* function through injecting morpholine-modified oligos results in an expansion in the caudal portion of the retina as well as a loss of the ventral retina and RPE (Figure 3). Instead, the ventral retina is replaced by *pax2.1* expressing cells.
These aberrant phenotypes in the eye that accompany lmo4 misexpression suggest that it might act as a negative regulator of retinal specification and vesicle outgrowth while acting as a positive regulator of retinal pigmented epithelium. The role of lmo4 in optic stalk specification is unclear.

**LIM domains**

Lmo4 belongs to the LIM-only class of proteins, because it consists almost entirely of 2 tandem LIM domains. The LIM domain was named such because it was initially identified as a common motif in three proteins:
Lin-11 (functions in asymmetric cell division in *C. elegans*), Islet-1 (functions in motor neuron development in *rat*), and Mec-3 (functions in touch receptor neuron differentiation in *C. elegans*) (Dawid et al., 1998). It consists of an approximately 55-residue cysteine-rich motif which contains two hydrophobic Zn$^{2+}$-binding domains (Dawid et al., 1998; Rabbitts, 1998). All LIM domains contain the consensus C-X$_2$-C-X$_{16-23}$-H-X$_2$-C-X$_2$-C-X$_2$-C-X$_{16-21}$-C-X$_{2,3}$-C/H/D-X$_2$ (Figure 4). Though the zinc fingers in LIM domains are structurally very similar to the zinc fingers in the DNA binding domains of GATA factors (Perez-Alvarado et al., 1996; Perez-Alvarado et al., 1994), LIM domains have not been shown to bind to DNA. However, they have been shown to mediate specific protein-protein interactions (Dawid et al., 1998; Rabbitts, 1998).

Proteins containing LIM domains are classified into three different groups (Dawid et al., 1995) based on the sequence of the LIM domains and the overall structure of the proteins containing them. Group 1 LIM proteins consist of Lhxs (LIM-homeodomain), Lmos (LIM only), and LIMKs (LIM kinases). These proteins contain a tandem of A and B class LIM domains at the N-terminus. Group 2 LIM proteins are composed largely of class C LIM domains which contain an additional conserved motif. Group 3 proteins are more diverse and contain a variable number of class D LIM domains at the
C-terminus. Group 1 proteins are predominantly nuclear, while group 2 and 3 proteins tend to be cytoplasmic. Since Lmo proteins belong to group 1, groups 2 and 3 will not be discussed further.

Within group 1, the LIMK proteins contain a kinase domain as well as 2 LIM domains. Lhx proteins are transcription factors which contain a DNA binding homeodomain in addition to the 2 tandem LIM domains at the N-terminus. Lmo proteins are transcriptional cofactors which consist almost entirely of 2 tandem LIM domains.

Figure 4: The consensus class 1 LIM domain structure.
Other classes vary with respect to the spacing between the zinc binding residues. Reprinted with permission from Dr. Mary Ellen Lane.

Figure 5: Schematic of Ldb.
LID=LIM interaction domain
Mechanisms of action of Lmo proteins

Functional analyses have revealed at least two different mechanisms of action for Lmo proteins (Figure 6). Firstly, they have been shown to antagonize the Lhx class of proteins (Milan et al., 1998), which are critical transcriptional regulators of embryonic development. Lhx proteins are only able to bind to DNA and activate gene transcription when they are bound to Ldb proteins (LIM domain-binding). Ldbs have a ~38 residue domain sufficient for LIM domain-binding at the C-terminus and a dimerization domain at the N-terminus (Jurata and Gill, 1997) (Figure 5). Binding to Ldbs allows the formation of the functional transcription factor complex which is a tetramer of two Ldb proteins and two Lhx proteins. Lmo proteins also bind to Ldbs, thereby sequestering them and competitively inhibiting Ldb-Lhx complex formation. Secondly, Lmo proteins have been shown to bind to bHLH factors, GATA factors (Rabbitts, 1998), DEAF (deformed epidermal autoregulatory factor) proteins (Sugihara et al., 1998), CtIP (adenovirus ElA C-terminal-binding protein), and the breast tumor suppressor BRCA1 (Sum et al, 2002) thereby acting as adapter molecules to facilitate the formation of multimeric transcription factor complexes.
**Structural analysis of Lmo-Ldb binding**

Recombinantly expressed Lmo and Lhx proteins tend to be very unstable and insoluble due to the hydrophobicity of the LIM domains and their tendency to bind non-specifically to other surfaces (Jurata et al., 1998; Deane et al., 2001). This had made the structural analysis of LIM domain-containing proteins extremely difficult. However, this problem had been circumvented by the production of a chimeric protein consisting of both LIM domains of human Lmo4, followed by a flexible 11-residue linker region, and the LIM interacting domain of human Ldb1 (Deane et al., 2004). This fusion protein forms a stable intramolecular complex (Figure 7) which covers the hydrophobic patch on the surface of Lmo4 and thus allows for the
production, purification, and structural analysis of the protein complex as well as the identification and characterization of the specific residues integral for the binding to occur.

![Diagram of Lmo4-LID chimera construct](image)

**Figure 7: Lmo4-LID chimera construct.**
The chimera construct was obtained by fusing the LIM domains of lmo4 with the LIM interaction domain (LID) of ldb1. Deane et. al, 2004.

The crystal structure of the intramolecular complex of this fusion protein revealed that the complex forms an extended, rod-like structure in which each of the LIM domains contributes 2 modules that bind the LIM interaction domain of Ldb1 (Deane et al., 2004; Figure 8).
Figure 8: Lmo4:Ldb1-LID structure. On top is a ribbon diagram of the structure. Ldb1-LID is shown in yellow. CCHC modules of Lmo4 are shown in cyan whereas CCCD modules of Lmo4 are shown in red. Below is a schematic representation of the topology of the complex.

Their data revealed that the interactions between both LIM domains of Lmo4 and the LIM interaction domain of Ldb1 are mediated by a combination of hydrogen bonds, hydrophobic interactions, and electrostatic interactions (Deane et al., 2004; Figure 9).
Figure 9: Schematic of interactions between LIM domains of Lmo4 and the LID of Ldb1. Ldb-LID residues are shown in yellow whereas interacting Lmo4 residues are shown either above or below and color-coded according the type of interaction: hydrophobic interactions shown in white, electrostatic interactions shown in red, side chain hydrogen bonds shown in light blue, and backbone-backbone hydrogen bonds shown in dark blue.

Qualitative yeast 2 hybrid assays making use of different selective markers with varying stringencies together with an ELISA based competition assay showed that the binding affinity between full-length Lmo4 and the LIM interaction domain of Ldb1 is at least 1000-fold greater than the binding affinity between N-terminal LIM domain of Lmo4 with the LIM interaction domain of Ldb1 (Deane et al., 2004). The binding affinity between the C-terminal LIM domain of Lmo4 with the LIM interaction
domain of Ldb1 is reported to be several fold weaker than that of the N-terminal LIM domain of Lmo4 (Deane et al., 2004).

**Candidate interactors with Lmo4 in eye development**

Because Lmo proteins contain two protein interaction LIM domains and are known to act through mechanisms involving interaction with other factors to modulate their transcriptional activity, we have searched the available literature for candidate interactors to Lmo4 that might mediate its role in zebrafish eye development.

**Ldb5**

There are 4 known zebrafish members of the *ldb* gene family. Ldb1, Ldb2, Ldb3, and Ldb4 share 95%, 73%, 62%, and 83% amino acid identity with mouse Ldb1, respectively (Toyama, 1998). Zebrafish Ldb4 is 86% identical to zebrafish Ldb1 at the amino acid level.

As previously mentioned, Ldb proteins are named such because they are LIM domain binding. At the C-terminus, they have a ~38 residue LIM interaction domain (LID) (Figure 5), and at the N-terminus is a ~200 residue homodimerization domain (Jurata and Gill, 1997). Functional analysis of
CHIP, the *Drosophila* homolog of Ldb/NLI (nuclear LIM domain interactor) and Ldb1 (*Xenopus* homolog of Ldb) have revealed their roles as essential cofactors to Lhx proteins (Agulnick et al., 1996; van Meyel, 1999). Ldb5s act synergistically with Lhx proteins by binding them at their LIM domains through LID motifs.

Expression studies have shown that all four known zebrafish *l dbs* demonstrate dynamic and variable expression patterns. However, all are expressed in the developing eye (Dawid et al., 1998). Therefore, all four zebrafish *l dbs* are candidate interactors.

**RLIM**

RING, Really Interesting New Gene, fingers are zinc fingers that have been identified in hundreds of genes involved in diverse cellular functions, including ubiquitination (Pickart, 2001). Ubiquitin protein ligases, or E3s, are involved in the polyubiquitination of proteins. Oftentimes, they contain RING fingers. Ubiquitin is 76-residue polypeptide that, when conjugated to cellular proteins, targets them for degradation either via an ATP-dependent protease known as the 26 proteasome or via a lysosome. Ubiquitination requires the actions of 3 different enzymes: E1 activating enzymes which activate ubiquitin, E2 conjugating enzymes that transiently carry activated...
ubiquitin as a thiol ester, and E3 ubiquitin ligases that transfer the activated ubiquitin from E2 proteins to substrate targets.

RLIM is a RING finger LIM domain-binding protein that has been described in mice, chick, and human as an ubiquitin protein ligase. It is expressed ubiquitously in early mouse embryos (E7). Functional studies of RLIM have shown it to be a corepressor of LIM domain-containing proteins and Ldbs by binding them and targeting them for degradation through ubiquitination (Ostendorff et al., 2002). Thus, any RLIM discovered in zebrafish would be a candidate interactor to Lmo4 as well as a potential regulator of Lmo4 protein accumulation.

Candidate targets for antagonism by Lmo4

Islet3,

Islet3 encodes an LIM-homeodomain transcription factor that is initially expressed ubiquitously throughout zebrafish embryos but gradually becomes restricted specifically to the eyes and the presumptive tectum in the CNS of 20-24 hour embryos (Kikuchi et al., 1997; Tokumoto, 1995). In order to elucidate its function, the LIM domains of islet3 were overexpressed with the rationale that they would act as a dominant-negative
variant of Islet3 by effectively binding and sequestering Ldb5 in a competitive manner, thereby knocking-down endogenous Islet3 function (Kikuchi et al., 1997). These experiments resulted in eyeless embryos in which the optic vesicles failed to evaginate and develop into eye structures. The specificity of the antagonism was confirmed by rescue experiments in which co-injecting full length islet3 could rescue the dominant/negative phenotype whereas coinjection of islet1 could not rescue. Based on the similarity between the phenotype obtained when just the LIM domains of Islet3 are overexpressed and the lmo4 overexpression phenotype, Islet3 is an attractive potential target for antagonism by Lmo4.

**Lhx2**

*Lhx2* is another LIM-homeodomain gene which is required for eye development (Porter et al., 1997). In mice, it is expressed in the telencephalon, diencephalon, and part of the myencephalon. Inactivating *lhx2* in mice results in a lack of lens, globe, and eyes due to arrest of eye development after formation of the optic vesicles but prior to the formation of the optic cups. This phenotype is similar to the eye phenotype found in small eye (*Sey*) mice which are mutant for *pax6*. However, when *pax6* expression was analyzed in *lhx2* mutant embryos, its expression was found
to be intact in the arrested optic vesicles, although \textit{pax6} expression in the overlying ectoderm was abolished. Conversely, \textit{lhx2} expression was found to be normal in \textit{Sey} mice. These results suggest that \textit{lhx2} and \textit{pax6} are both essential in an independent manner for proper development of the optic vesicles and optic cups. Given its knock-out phenotype, Lhx2 represents another possible target of antagonism by Lmo4.

\textbf{Zebrafish as a model organism}

The greatest progress in understanding the molecular mechanisms of eye development has been made in \textit{Drosophila melanogaster} due to the power of its molecular genetics. However, the insect eye is structurally and functionally very different from the vertebrate eye. This has led many to look for new vertebrate model organisms in which similar rapid genetic screens can be performed. Zebrafish (\textit{Danio rerio}) has emerged as an ideal vertebrate model for development with its small size, ease of care, rapid generation time, accessibility of early embryonic stages, the transparency of the early embryo, and available molecular genetic methodology including transgenesis, ease of overexpression through microinjection, and reverse genetics through the use of morpholino oligonucleotides.
Morpholino antisense oligonucleotides were first introduced into developmental biology in 2000 (Heasman, 2002). Since then, they have been used in a variety of model organisms including zebrafish (Nasevicius, 2000). Conventional antisense RNA or antisense DNA-mediated knock-down of gene expression has been unfruitful in zebrafish due to non-specific side-effects (Oates et al., 2000). Morpholino oligonucleotides are DNA analogs in which the sugar is replaced by a morpholine group (C$_4$H$_9$NO) (Summerton and Weller, 1997). These oligonucleotides function through an RNAse H-independent mechanism. When designed to be complementary to 5' leader sequences or to the first 25 bases of the open reading frame (ORF), they block translation of the mRNA by preventing ribosomes from binding and initiating translation. Their specificity has also been proven in that as little as a 4 base pair mismatch can render morpholino oligonucleotides ineffective. In theory, you can knock-down any gene in zebrafish using morpholino oligonucleotides, and you can knock-down multiple genes at once using multiple morpholino oligonucleotides.

Zebrafish is also a particularly ideal model for studying vertebrate eye development. Its eyes are larger relative to body size than mammalian eyes, and zebrafish eyes develop rapidly such that by 24 hours post fertilization, the morphology of the eyes is complete.
Eye development in zebrafish occurs in stages

The process of eye development in zebrafish can be thought of as occurring through a series of inductive events and morphogenetic cell movements.

Neural induction and forebrain specification

In zebrafish, axial mesoderm consisting of the prechordal plate (the precursor to head mesoderm) and the chordamesoderm (precursor to the notochord) secrete paracrine factors such as Chordin (Sasai et al., 1994), which antagonize bone morphogenic proteins (BMPs) made in the ventral and lateral regions of the embryo that normally induce the overlying ectoderm to become epidermis (Barth et al., 1999). As a result, an area of dorsal ectoderm is specified to become the neural ectoderm (Figure 10A) which will eventually give rise to the brain and spinal cord. After neural specification, Wnt antagonists are responsible for the specification of the forebrain in the anterior neural plate. Mutations affecting signal transduction via the Wnt pathway such as mutations of the masterblind and headless loci abolish both optic and telencephalic fates, while midbrain fates are expanded rostrally (Heisenberg et al., 1996; Kim et al., 2001).
Specification of the optic primordia

Once the anterior forebrain has been specified, fate mapping (Woo, 1995); (Varga, 1999) and gene expression studies (Chuang, 1999; Chuang, 2001) show that a single optic anlage is specified within the medial rostral neural plate (Figure 10B) at the end of gastrulation. Functional studies show several genes are involved in this specification event. The retinal homeobox genes rx1 and rx2, pax6 (paired homeobox), and six3 (SIX-homeodomain) all appear to be key players in specification of the eye field. During neurulation in zebrafish (the formation of the neural tube), ventral diencephalic precursors migrate to the anterior effectively splitting the single eye field into two, bilateral optic vesicles (figure 10B-C), which migrate to the periphery and evaginate from the forebrain.

Specification of different cell types within the optic primordia

Finally, the posterior part of the vesicles invaginate to form the optic cup and proximodistal patterning of the optic primordia occurs (Figure 10D). By 24 hours, the optic cup (retina) consists of 2 cell layers: the pax6 expressing retinal neuroepithelium (NE) and the mitf expressing retinal pigmented epithelium (RPE). The point of attachment of the optic cup to the
forebrain becomes the pax2 expressing optic stalk, which forms the trajectory for the optic nerve. By 3 days, retinal neurogenesis is nearly complete, and the eye becomes functional.

Functional studies suggest that fibroblast growth factor (FGF) signaling from the surface ectoderm and hedgehog signaling from the midline are involved in the patterning of different cell types along the proximodistal axis of the optic primordia. Specifically, hedgehog induces proximal stalk fates, while FGF signaling induces a distal optic cup fate. The specification of the RPE requires the microphthalmia transcription factor (mitf) pathway.

**Figure 10. 4 steps of eye development.**
A. Induction of the neural plate (gray) B. Induction of a single eyefield within the anterior neural plate (blue). C. Splitting of the eyefield into 2 optic vesicles which evaginate from the forebrain (purple) D. Formation of the optic cup and specification of the different cell types within the optic primordial: optic stalk (red), RPE (blue-green), and NR (yellow). Figure reprinted with permission from MEL.
MATERIALS AND METHODS

Design of islet-3 morpholino oligonucleotide

Antisense morpholino oligonucleotides targeted to the 5’UTR and spanning the ATG of islet3 were designed by Gene Tools, Inc., based on submission of sequence spanning the interval from -75 to +25 of the islet3 cDNA. The morpholino oligonucleotide is directed against a region of the islet3 transcript spanning -19 to +6. The morpholino oligonucleotide sequence is CACCATAACGTTGCCCCGAAGATTG. A negative control morpholino oligonucleotide including 5 base pair mismatches was also designed. This morpholino oligonucleotide sequence is CAgCAaACAGGTTcCCCCcAAcATTG. A .1 mg/mL stock was made for each morpholino oligonucleotide. The following dilutions were made in .5X MBS + .5% phenol red for injection: 3ng/5nL, 6ng/5nL, 9ng/5nL, and 12 ng/5nL. 5nL of these injection solutions were delivered to each embryo.

Making RNA including 5’ UTR encoding a C-terminal myc-tagged variant of Islet3

Islet3 ORF + the entire 5’ UTR was cloned into pCS2myc using primers DJ 61 and DJ 62 and the restriction sites BamH1 and Cla1. RNA
was made by cutting the construct with Not1 and transcribing with the SP6 message machine kit. This RNA was used to perform the Islet3 rescue experiment. We also obtained a construct from the Okamoto lab (Kikuchi et al., 1997) in which the full-length cDNA of Islet3 was cloned into pCS2+ using the restriction enzyme sites BamH1 and EcoR1. A third construct, pSP64T Isl3 was also made in which the ORF of Islet3 was clones into pSP64T using primers DJ44 and DJ45 and the restriction enzyme sites BglII and EcoRV. Injecting RNA made from each of the three different expression constructs resulted in embryo abnormalities including embryo truncation as well as defects in eye defects. This is in stark contrast to reports from the Okamoto lab which claim islet3 overexpression through injection of as much as 1.4 ng of RNA might cause embryo truncation but does not affect eye development.

To confirm the integrity of the RNAs injected, RNA injection solutions were checked on a gel immediately after the injections were performed (data not shown). Additionally, animal caps were cut from embryos injected with RNA encoding myc-tagged Islet3 and checked via western blot analysis using antibodies against the myc epitope to verify protein accumulation (data not shown).
Cloning of yeast 2-hybrid constructs

Zebrafish *lmo4* was cloned into pGBK T7 (Clontech) using primers DJ12 and DJ13 and the restriction enzyme sites Nco1 and BamH1. The N-terminal LIM domain of *lmo4* was cloned into pGBK T7 using primers MEL110 and MEL 130 and the restriction enzyme sites Nco1 and BamH1. The C-terminal LIM domain of *Lmo4* was cloned into pGBK T7 using primers MEL 131 and MEL 111 using the restriction enzyme sites Nco1 and BamH1. The C48G mutation in *lmo4* was made using mutagenic primers MEL140b and MEL 141b using the restriction enzyme sites Nco1 and EcoR1. The C113G mutation was made using mutagenic primers MEL144 and MEL 145 and the restriction enzyme sites Nco1 and EcoR1. The D77H mutation was made using mutagenic primers MEL142a and MEL143a and the restriction enzyme sites Nco1 and EcoR1. The D141H mutation was made using mutagenic primers MEL 146 and MEL 147 and the restriction enzyme sites Nco1 and EcoR1. *Ldb1* was cloned into pGAD T7 using primers DJ 12 and DJ 13 using the restriction enzyme sites Nde1 and BamH1. *Ldb2* was cloned into pGAD T7 using primers DJ 14 and DJ 15 using the restriction enzyme sites Nde1 and BamH1. *Ldb3* was cloned into pGAD T7 using primers DJ 32 and DJ33 using the restriction enzyme sites Nde1 and BamH1. *Mitfa* was cloned into pGAD T7 using primers DJ36 and
DJ37 using the restriction enzyme sites Nde1 and EcoR1. Mitfβ was cloned into pGADT7 using the primers DJ38 and DJ39 using the restriction enzyme sites Nde1 and BamH1.

Controls were performed as follows. As a positive control, we have a plasmid which encodes for full-length GAL-4 (pCL1). Also, we have plasmids which encode for p53 fused to the DNA-BD (p53-BD) domain of GAL-4 and T antigen fused to the AD domain of GAL-4 (T-AD). p53 and T antigen are known interactors. Both full-length GAL-4 and p53-BD together with T-AD activate reporter gene expression. As a negative control, we have a construct encoding a fusion between human laminin C and the DNA-BD of GAL-4 (Lam-BD), which should not interact with any of our proteins. Also, single transformations of each of our 2-hybrid constructs were performed as negative controls. Thus far, the only aberrant results are those obtained with yeast singly transformed with a construct encoding the Isl3-BD fusion. There appears to be some autoreactivity associated with this construct.

Ldb1, ldb2 and ldb3 were obtained as a generous gift from Dr. Reiko Toyoma (Toyoma et al., 1998) at the NIH. Yeast 2 hybrid experiments were performed using the Y187 strain which contains a lacZ reporter gene under the control of the yeast GAL4 promoter.
Yeast transformation

Yeast 2-hybrid constructs were transformed into Y187 using the small scale transformation method provided by the kit. Double transformations were plated on –Leu/-Trp double selection media.

Qualitative colony lift filter assay

The qualitative colony lift filter assay was obtained from the yeast protocols handbook accompanying Clontech's Matchmaker yeast 2 hybrid system. Y187 yeast cultures were transformed with the yeast 2 hybrid constructs to be tested and plated on selective media. Cells were grown for about 4 days. Z buffer was prepared by mixing 16.1 g/L of Na₂HPO₄*7H₂O, 5.5 g/L NaH₂PO₄*H₂O, 0.75 g/L KCl, and 0.246 g/L MgSO₄*7H₂O. The solution was adjusted to pH 7.0 and autoclaved. X-gal stock solution was prepared by dissolving X-gal in N,N-dimethylformamide at a concentration of 20 mg/mL. For each plate of transformants to be assayed, sterile Whatman filters were soaked in 2.5 mL of Z buffer/X-gal solution (100mL Z buffer, 0.27 mL β-mercaptoethanol, 1.67mL X-gal stock solution) in a 100 mm plate. Forceps were used to place a clean, dry filter over the surface of the plate of colonies. After the filter becomes evenly wetted, it was lifted off
of the plate and transferred to a pool of liquid nitrogen and submerged for 10 seconds. This lyses the cells. The filter was then removed from the liquid nitrogen and placed colony side up on the presoaked filter and allowed to incubate at room temperature for about 1 hour. After incubation, filters were checked for the presence of blue colonies which signals an interaction between the proteins assayed.

**Quantitative β-galactosidase assay**

The quantitative β-Gal assay was obtained from the McNew lab (James McNew, unpublished). This assay quantitatively compares the amount of LacZ reporter expressed in the yeast culture through the use of a substrate for LacZ, FDG. FDG is a fluorescein molecule attached to a sugar. When cleaved by LacZ the fluorescein becomes active and its presence can be detected through the use of a fluorimeter. A 5 mL culture was seeded with an individual colony and grown overnight to mid-log phase (OD 600~0.5~0.8) at which point the cultures were diluted in media to reach on OD of exactly 0.5. 100 μL of the culture was pipetted into 3 different wells of a 96 well plate. 20 μL of an FDG solution containing triton X-100 was added to each well (83μM fluorescein di-β-D-galactopyranoside [10mM stock in dimethyl sulfoxide], 137.5 mM PIPES pH 7.2, 2.5% Triton X-100).
The Triton detergent permeabilizes the cells without removing the cell walls, while the FDG serves as a substrate for β-Galactosidase that can be detected after cleavage. Plates were covered with parafilm and aluminum foil and allowed to incubate for 90 minutes at 37 C with shaking. Upon completion, the plate was read using a fluorimeter with an excitation wavelength of 465nm and an absorption filter of 538 nm wavelength. (Note: the 465nm excitation filter is not the optimal filter but was used nonetheless due to problems with the 485nm filter).

Making yeast extracts for Western blot analysis

The procedure for making yeast extracts was obtained from the McNew lab. Single colonies were used to inoculate a 20 mL starter culture that was allowed to incubate at 30 C with shaking for 2 days. The starter culture was then added to 100 mL of fresh media to reach an OD 600 of ~0.3. This final culture was incubated at 30 C with shaking for ~8 hours until the OD 600 was at least 0.5 at which point 50 OD cells were harvested. Cells were resuspended in 1 mL of water and transferred to microfuge tubes. Cells were spun down and resuspended in 1mL 5% TCA and incubated on ice for 1 hour. Cells were spun down and resuspended in 1mL of water. Cells were spun down and resuspended in 250 µL of 2X Laemelli buffer
without bromophenol blue. 20 μL of 1N NaOH was added. The culture was transferred into to 10X75 mm glass tubes containing 0.3 g of acid-washed glass beads (Sigma G-8772). Tubes were covered with parafilm and vortexed for 20 seconds 6 times and then boiled for 10 minutes. The extract was transferred back into microfuge tubes and spun for 5 minutes at top speed. The extract was recovered to a new microfuge tube. 5 μL of this extract was used for Western blot analysis.

Making RNAs encoding C-terminal myc-tagged mutant forms of Lmo4

WT lmo4, the N-terminal LIM domain of lmo4, the C-terminal LIM domain of lmo4, the lmo4 double mutant (C48G, C113G), lmo4-C48G, lmo4-C113G, lmo4-D77H, and lmo4-D141H were all cloned into pCS2MT. pCS2MT N-myc Lmo4 was cloned by Catherine McCollum. RNA was made by cutting with Not1 and transcribing with SP6 RNA polymerase. pCS2MT N-myc LIM N and pCS2MT N-myc LIM C were cloned by Ron Brannen. RNA was made by cutting both with Not1 and transcribing with SP6 RNA polymerase. pCS2MT lmo4-C48G, pCS2MT lmo4-C113G, pCS2MT lmo4-D77H, pCS2MT lmo4-D141H, and pCS2MT lmo4-C48G,C113G were made using the same mutagenic primers used in making the yeast 2-hybrid mutant constructs and cloned into pCS2 using the
restriction enzyme site EcoR1. RNA was made by cutting constructs with NotI and transcribing with SP6 RNA polymerase.

Making RNA including 5' UTR encoding a C-terminal myc-tagged variant of Islet3

*Islet3* ORF + the entire 5’ UTR was cloned into pCS2myc using primers DJ 61 and DJ 62 and the restriction sites BamH1 and ClaI. RNA was made by cutting the construct with NotI and transcribing with the SP6 message machine kit. This RNA was used to perform the Islet3 rescue experiment. We also obtained a construct from the Okamoto lab in which the full-length cDNA of Islet3 was cloned into pCS2+ using the restriction enzyme sites BamH1 and EcoR1 (Kikuchi et al., 1997). A third construct, pSP64T Isl3 was also made in which the ORF of Islet3 was clones into pSP64T using primers DJ44 and DJ45 and the restriction enzyme sites BglII and EcoRV. Injecting RNA made from each of the three different expression constructs resulted in embryo abnormalities including embryo truncation as well as defects in eye defects. This is in stark contrast to reports from the Okamoto lab which claim Islet3 overexpression through injection of as much as 1.4 ng of RNA might cause embryo truncation but does not affect eye development.
Making the DDHD fusion construct

The dimerization domain of LDB1 was amplified using primers MEL 165 and MEL 166.6 and subcloned into CS2 myc using the restriction enzyme sites EcoR1 and Xba1. Isl3 HD was cloned using primers MEL 167 and MEL 168 and inserted into CS2myc DD using the restriction enzyme sites Afe1 and Xba1. RNA was made by cutting the construct with Not1 and transcribing with SP6 RNA polymerase.

Making a probe for zebrafish rlim

The mouse rlim sequence was blasted against zebrafish genomic shotgun sequence. This produced a ~300 bp match with an e-value of 1e-34. This partial sequence has high homology with the mouse RLIM protein from residues 498 to 593, which includes the RING finger consisting of residues 546 to 586. This fragment was cloned into pBS using primers DJ46 and DJ47 and the restriction enzyme sites EcoR1 and Spe1 for probe making. Additionally, primers DJ48 and DJ49 were designed for 5' and 3' RACE PCR to clone full length rlim cDNA.
RESULTS

Attempted rescue of *lmo4* gain of function phenotype by ectopic overexpression of *islet3*

As described in the background, experiments by Hitoshi Okamato’s laboratory implicate *islet3* in eye development (Kikuchi et al., 1997). They showed that overexpressing a dominant-negative variant of the protein consisting of only the LIM domains of *Islet3* resulted in an eyeless phenotype similar to the *lmo4* overexpression phenotype (Fig 2). These results strongly suggest that *islet3* could be a target for antagonism through competitive inhibition by *lmo4*. To explore this possibility, we attempted a rescue of the *lmo4* gain of function phenotype by coinjection of full length *islet3* mRNA. If Lmo4’s role in eye development is to prevent retinal specification in the presumptive RPE and optic stalk by antagonizing the activity of Islet3 by binding and sequestering Ldb cofactors, then overexpressing *islet3* should rescue the Lmo4 gain of function phenotype by restoring the equilibrium. *Islet3* activity should be rescued by simply adding more of it. So, if we co-inject embryos at the 1 cell stage with RNA encoding Lmo4 and Islet3, we would expect to see normal embryos. We
should be able to at least partially rescue the *lmo4* gain of function eyeless/small eye phenotype.

To this end, we co-injected *myc-islet3* mRNA along with *myc-lmo4* mRNA into 1-2 cell stage embryos and observed them for eye phenotypes at prim5 stage (28 hpf). If our hypothesis is true, we would expect the co-injection of *islet3* mRNA to result in a rescue of the eye phenotype associated with *lmo4* mRNA injection.

Four different sets of rescue experiments were undertaken with respect to the amount of *lmo4* RNA injected to obtain an eye phenotype. We first attempted to rescue the phenotype obtained with injecting 60 pg of *lmo4* RNA. Additionally 120 pg, 250 pg, 400 pg, and 550 pg of *lmo4* RNA was injected. Within each set of injections, either 250 pg, 500 pg, or 750 pg of *islet3* RNA was co-injected with the *lmo4* RNA to ascertain whether rescue could occur. For each set of experiments, rescue was not achieved (Table 1, Figure 11).

Embryos injected with *lmo4* RNA and *gfp* RNA predominantly exhibited characteristic small eye or eyeless phenotypes. The *lmo4* overexpression eye phenotype was not rescued by *islet3* coinjection. Indeed, *islet3* coinjection often resulted in enhancing the severity of the eye phenotype. Control experiments were performed in which either 250, 500,
750 pg *islet3* RNA alone was injected into embryos at the 1-2 cell stage. Injecting 250pg of *islet3* RNA resulted in the lysis of ~80% of the injected embryos during gastrulation. The surviving embryos developed into 24 hour old larvae with severe developmental abnormalities including, but not limited to, dorsalization, body truncation, flattened head, and a lack of eyes. Injecting 500 pg and 750 pg of *islet3* RNA alone resulted in an even higher frequency of embryo lysis. This is in contrast to reports by Kikuchi et al. stating that injection of as much as 1.4 nanograms of *islet3* mRNA did not adversely affect eye development although body truncation was frequently witnessed with injections of such high amounts. The embryo lysis that was evidenced with *islet3* overexpression was mitigated by coinjection of *islet3* RNA with *lmo4* RNA. Only approximately 5% of embryos coinjected with *islet3* RNA and *lmo4* RNA lysed. These results suggest that *islet3* overexpression can not rescue the *lmo4* overexpression eyeless phenotype, and that *islet3* may not be a target for antagonism by *lmo4* in eye development. However, the fact that overexpressing *lmo4* can rescue the gastrulation defects caused by *islet3* overexpression suggest that the two may act antagonistically during gastrulation.
<table>
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Table 1: islet3 coinjection can not rescue {iso}4 overexpression phenotype.
Each row represents an injection experiment. Column 1 denotes the RNAs injected into embryos at the 1-2 cell stage. Embryos were allowed to develop until 24 hours post fertilization and scored on eye phenotype alone. Results are shown as a percentage of total embryos assayed for each injection experiment.
Figure 11: *islet3* coinjection can not rescue *lm04* overexpression phenotype. This is a bar graph representation of the No Eyes results in Table 1. The X-axis specified the combination of RNAs injected. The Y-axis displays the percentage of injected embryos that lacked eyes altogether at 24 hours post fertilization.
Knock down of *islet3*

Interestingly, Hitoshi Okamato’s group has not reported an *islet3* knock down phenotype in zebrafish. In order to confirm the hypothesis that *islet3* is necessary for eye development in zebrafish, we conducted *islet3* loss of function experiments using morpholine-modified antisense oligonucleotides. We designed antisense morpholino oligonucleotides targeted to the 5’UTR and spanning the ATG of *islet3*. Preliminary injections suggested that our morpholino oligonucleotide might be ineffective. Microinjection of up to 12 nanograms of morpholino oligonucleotide results in a subtle phenotype in the midbrain-hindbrain boundary, but not in the eyes. We would expect *islet3* morphant embryos to display microphthalmia similar to the $LIM^{isl3}$ overexpression phenotype if it is indeed involved in eye development. However, the eyes of morphant embryos look normal. Thus, to determine whether our morpholino oligonucleotide is effective at the molecular level, we designed an experiment to test the morpholino oligonucleotide’s ability to block the translation of exogenous *islet3* transcript.

To this effect, we made an expression construct including the 5’UTR of *islet3* along with its ORF attached to sequence encoding the myc epitope tag. RNA made from this construct was co-injected along with our *islet3*
morpholino oligonucleotide into zebrafish embryos at the one cell stage. Embryos were allowed to develop to the blastula stage (~4 hours post fertilization) at which time animal caps were cut for western blot analysis against the myc epitope.

This experiment demonstrated that our *islet3* morpholino oligonucleotide was effectively preventing the accumulation of exogenous myc-tagged Islet3 protein (Figure 12) at least until the blastula stage.

*Figure 12. islet3 morpholino oligonucleotide blocks Islet3myc protein accumulation.*

1 cell stage embryos were either uninjected, injected with *islet3myc* RNA alone (250 pg), or co-injected with *islet3myc* RNA (250 pg) and different amounts of either *islet3* morpholino oligonucleotide or a control morpholino oligonucleotide with 5 mismatches incorporated into it that should render the morpholino oligonucleotide ineffective. Animal caps were cut from blastula stage embryos and analyzed via Western blot analysis against the myc epitope tag for Islet3myc protein accumulation.
In order to determine whether the action of the morpholino oligonucleotide could persist to later time points, we repeated the experiment, only this time we made extracts from larvae that were permitted to develop for 24 hours after injection (Figure 13). These experiments revealed that our morpholino oligonucleotide’s activity does persist at least until the 24 hour post fertilization stage. Therefore, our morpholino oligonucleotide seems to be effective at blocking islet3 transcription at the molecular level.

Figure 13. islet3 morpholino oligonucleotide activity persists to 24 hpf.
The experiment in figure 11 was essentially repeated, the only difference being that some of the injected embryos were allowed to develop until 24 hours post fertilization for analysis.
However, the injection up to 12 ng of *islet3* morpholino oligonucleotide does not produce an observable eye phenotype. We observe an extremely subtle phenotype at the midbrain-hindbrain boundary in contrast to the severe MHB phenotype reported by Kikuchi and colleagues during their dominant/negative experiment (Kikuchi et al., 1997). This suggests that *islet3* may not be necessary in the developmental process of early eye development in zebrafish.

**Identification and characterization of Lmo4-Ldb protein interactions**

The literature demonstrates that Lmo members function through protein-protein interactions. To address the question of mechanistically how Lmo4 protein might be carrying out its role in eye development, we sought to identify and characterize interactions involving Lmo4 using the yeast 2-hybrid system.

The yeast 2-hybrid system provides a transcriptional assay for detecting protein-protein interactions *in vivo* in yeast. It’s based on the yeast GAL-4 transcription factor, which encodes an enzyme needed to utilize galactose as a carbon source. A bait gene is expressed as a fusion to the GAL-4 DNA binding domain, while a prey gene or cDNA library is expressed as a fusion to the GAL-4 activation domain (Chien et al., 1991;
Fields and Song, 1989). Consequently, when the bait and prey genes interact, the DNA-BD and AD of GAL-4 are brought into close proximity resulting in the activation of reporter genes under the control of GAL-4 promoters.

We utilized Clontech’s MATCHMAKER GAL4 Two-Hybrid System 3 kit, which makes use a lacZ reporter under the control of the GAL-4 promoters. Zebrafish ldb1, ldb2, and ldb3 were cloned into the yeast 2 hybrid system for testing. Ldb4 is very similar to ldb1. Their sequences are 86% identical at the amino acid level. Efforts to obtain a full length ldb4 cDNA from third parties were unsuccessful. Therefore, rather than investing the energy to clone a full-length ldb4 cDNA for cloning into the 2 hybrid system, ldb4 was not tested in our analysis of Lmo4-Ldb interaction. Given it’s similarity to ldb1, we predict that the protein encoded by the ldb4 locus would interact with Lmo4 in a similar fashion as Ldb1. Yeast 2-hybrid constructs were transformed into the Y187 yeast strain using the small scale yeast transformation protocol provided by the kit. Interactions between bait and prey fusions were assayed using a quantitative β–Gal assay which uses the Triton X-100 detergent to permeabilize yeast cells and fluorescein di-β–D-galactopyranoside which is a substrate for β–galactosidase that is cleaved into fluorescein and detected through a fluorimeter.
We have determined that Lmo4 can interact with zebrafish Ldb1, 2, and 3 (Figure 14). However, the interaction is strongest with Ldb1 and weakest with Ldb3. In order to determine which LIM domain of Lmo4 might be mediating Lmo4-Ldb interactions, we made point mutations in conserved zinc coordinating residues within either the N terminal LIM (C48G) or the C terminal LIM (C113G). Mutating these residues should abolish the structure, and therefore function, of the LIM domains harboring these mutations. When we mutated the N terminal LIM domain (hereafter referred to as LIM A), it was still able to bind to Ldb1, 2, and 3. Interestingly, mutating LIM A resulted in a stronger interaction with Ldb1. Similarly, when we mutated the C terminal LIM (hereafter referred to as LIM B), the construct was still able to interact with all three Ldbs and mutating LIM B strengthened Lmo4’s interaction with Ldb1. When we tested the double Lmo4 mutant, the interaction was blocked. This data suggests that either LIM domain of Lmo4 is able to mediate Lmo4-Ldb interactions. In order to confirm this result, we proceeded to test individual, isolated LIM domains in the 2 hybrid system. These experiments revealed that both LIM A and LIM B of Lmo4 were indeed able to mediate interactions with the zebrafish Ldbs. Interestingly, the interaction between LIM B and the Ldbs is statistically stronger than that between LIM A and
the Ldbs. Next, we mutated individual LIM domains with the same mutations that we used earlier to test the efficacy of abolished LIM domain structure and function. Mutating isolated LIM A with a C48G mutation and mutating isolated LIM B with a C113G mutation resulted in abolishing the individual LIM domains’ ability to bind Ldbs. Thus, our point mutations appear to be effective at disrupting the structure of the LIM domains harboring them. Both LIM domains of Lmo4 have an aspartic acid residue at the last zinc-coordinating position. Other classes of LIM domain containing proteins can have either a histidine or a cysteine at that position. However, nuclear LIM domain containing proteins have a highly conserved aspartic acid residue. Its conservation suggests some functional significance with respect to that residue. So we decided to mutate that aspartic acid into a histidine in either LIM A (D77H) or LIM B (D141H). The mutation should create a stronger, more robust zinc finger, because the nitrogen on histidine should bind the Zn$^{2+}$ ion more tightly than the oxygen in aspartic acid. Both Lmo4 D77H and Lmo4 D141H were able to interact with all 3 Ldbs and the strengths of the interactions were comparable to that between wild type Lmo4 and the Ldbs. Thus, we were not able to uncover the functionality of the aspartic acid residues through the 2 hybrid system. However, we can go
back into the embryo through overexpression studies to determine their functional significance.

Additionally, qualitative colony lift filter assays were performed which revealed that Ldb1, Ldb2, and Ldb3 can all heterodimerize, which hasn’t yet been reported in the literature. Also, Islet3 can interact with Ldb1, Ldb2, and Ldb3.

**Figure 14: Summary of quantitative yeast 2 hybrid results.** The Y-axis displays interaction strength as a percentage of the interaction strength of p53 and T antigen, which was used as a positive control. The x-axis shows the different interactions assayed. The average and standard deviation of the results is shown for 3 independent transformations. Each independent experiment was performed in triplicate. Thus the statistical analysis was performed on 9 different measurements. The interaction strength between p53 and T antigen was used as a positive control, while the known lack of interaction between laminin and T antigen was used as a negative control.
It is important for us to show for each and every result that the fusion proteins are indeed being expressed at comparable levels. This would rule out a variation in the strength of the results due to variations in expression levels of the proteins being tested. Thus for every experiment in chart 1, we have made yeast extracts of yeast transformed with the corresponding bait and prey fusions for western blot analysis to detect protein expression. The bait fusion protein is tagged with a myc epitope, while the prey fusion is tagged with an HA epitope for detection. These experiments showed comparable expression levels for all interactions assayed (data not shown).

_Lmo4 mutant gain of function analysis_

Our 2-hybrid experiments have uncovered some important characteristics of Lmo4-Ldb interactions. Specifically, both LIM domains are able to mediate an interaction between Lmo4 and Ldb. Lmo4 binds more strongly to Ldb1 than to the other Ldbs. Also, LIM B of Lmo4 appears to interact more strongly than LIM A. Nevertheless, these studies do not tell us anything about what is necessary and sufficient for Lmo4 activity _in vivo_. Now that we've characterized interactions involving Lmo4 in the 2-hybrid system, we can take that information back into the embryo by overexpressing mutant forms of Lmo4 and assaying phenotypes. The WT
lmo4 gain of function small eye (or no eye) phenotype is robust. We can compare our mutant gain of function phenotypes to the lmo4 gain of function phenotype and use eye development as a bioassay for protein functionality. This information combined with the 2-hybrid interaction data would hopefully provide us with information as to which Lmo4 interactions might be relevant in eye development. In addition, it would be interesting to see whether different LIM domains of Lmo4 or different molecular interactions involving Lmo4 might be important in different developmental roles of lmo4. These types of experiments will not only tell us what is important for lmo4s role in eye development, but it should also elucidate important interactions necessary or sufficient for its role in pigmentation and craniofacial development as well.

To this effect, we injected embryos at the 1-2 cell stage with RNA encoding LIM A of Lmo4, LIM B of Lmo4, or Lmo4 C48G, C113G double mutant. In order to account for differences in the size of the RNAs injected and control injections as far as molar amounts of RNA injected, 1 ng of RNA encoding WT Lmo4 and Lmo4 C48G, C113G was injected. In contrast, only 750 pg of RNA encoding the deletion mutants was injected. Embryos were allowed to develop until prim5 stage (~28hpf) and eye phenotypes were assayed through measurements. Embryos were fixed and
mounted on glass slides. Pictures of the eyes were taken laterally and measurements were made from anterior to posterior through the center of the lens (Figure 15).

Wild type prim5 stage zebrafish embryos have eyes that are ~110 +/- 10 μm long (n=11). Overexpressing gfp results in no statistical difference with respect to eye size. Gfp gain of function mutants had an average eye length of 103 +/- 8 μm (n=30). Likewise, overexpressing the double mutant of lmo4 (lmo4 C48G, C113G) and overexpressing LIM A of lmo4 do not result in statistically significant changes in eye size. Double mutant gain of function embryos had an average eye length of 96 +/- 7 μm (n=59), while LIM A gain of function mutants had an average eye length of 95 +/- 14 μm (n=55). However, overexpressing LIM B of lmo4 results in a small eye phenotype. These embryos had eyes that are an average of 72 +/- 24 μm in length (n=65). Injecting the same molar amount of full length WT lmo4 RNA resulted in an eyeless phenotype in all 36 embryos injected (Figure 16).

As a control for protein accumulation, animal caps were cut from 10 injected embryos at 3 hours post fertilization for analysis via Western blot. All variants of Lmo4 protein assayed accumulated comparably (data not shown).
Figure 15: Representative samples of mutant lmo4 overexpression analysis. Embryos were injected at the 1-2 cell stage with the RNA indicated in the lower right hand corner and allowed to develop until 28 hours post fertilization at which point they were fixed and mounted for eye measurements. In order to control for the molar amount of RNA injected into embryos, 750 pg of gfp, lmo4, and lmo4-C48G, C113G RNA were injected whereas 1 ng of RNA encoding LIM-A and LIM-B were injected.
Figure 16: Summary of Lmo4 mutant eye phenotype analysis.
The Y-axis denotes average eye length in μm. The x-axis shows the different RNAs injected. Injected embryos were allowed to develop until 28 hours post fertilization at which point eye measurements were made and analyzed statistically. WT embryos had an average eye length of 110 nm (n=11). Gfp-injected embryos had an average eye length of 103 nm (n=103). Lmo4-injected embryos had an average eye length of 96 nm (n=59). Lmo4-C48G,C113G double mutant injected embryos had an average eye length of 96 nm (n=59). Lim A-injected embryos had an average eye length of 95 nm (n=55). Lim B-injected embryos had an average eye length of 72 nm (n=72).
DISCUSSION

We have initiated an analysis of the mechanism of action of Lmo4 with respect to the process of eye development in zebrafish. We have tested our hypothesis that lmo4 regulates eye development through an antagonism of islet3 activity. We have also identified and characterized Lmo4-Ldb interactions using yeast two hybrid and begun a structure-function study by examining the phenotype of embryos in which different variants of lmo4 are overexpressed. Overexpressing full-length lmo4 results in a small eye or eyeless phenotype depending on the amount of mRNA injected into embryos. Conversely, knocking down lmo4 activity through morpholine modified oligonucleotides results in an expansion of the retina. The robust eye phenotypes seen with manipulated levels of lmo4 expression present a powerful bioassay that can be utilized to study lmo4 structure-function relationships. This data will contribute to the general understanding of LIM domain-containing proteins and their mechanisms of action.

Islet3 may not play a role in zebrafish early eye development

Evidence from other laboratories suggests that islet3 which encodes a protein belonging to the LIM-homeodomain class of proteins may be
involved in zebrafish eye development. We have begun to test the hypothesis that the role of *lmo4* in eye development might be mediated by an antagonism of *islet3* activity. High levels of *lmo4* expression in medial regions of the optic cup may serve to block retinal differentiation and either allow or induce the differentiation of those regions into either optic stalk or retinal pigmented epithelium through an antagonism of *islet3* activity. Conversely, a lack of *lmo4* expression in the presumptive neural retina would allow Islet3 activity to induce the expression of retinal specific genes.

In order to test our model of Islet3 antagonism by Lmo4 in the optic cup, we have performed two experiments. First, a morpholino oligonucleotide directed against *islet3* has been designed and injected in order to determine whether *islet3* is truly involved in zebrafish eye development. Through these experiments, we were unable to obtain an *islet3* morphant phenotype. However, the efficacy of the *islet3* morpholino oligonucleotide at the molecular level up to 24 hours post fertilization was verified through the lack of exogenous protein accumulation that could be detected through Western blot analysis. Secondly, we attempted to rescue the *lmo4* gain of function phenotype with simultaneous coinjection of *islet3* mRNA. Again, these results were unsuccessful. Rescue was not achieved. In fact, coinjection often resulted in enhancing the eye phenotype.
These results can be interpreted in at least two different ways. First of all, *islet3* may indeed be involved in eye development, but the presence of a second redundant *islet3* ortholog in zebrafish may preclude the detection of a morphant phenotype. Studies in comparative genomics suggests that the ancestors of teleost fish underwent an extra round of genome duplication about 100 million years ago (Taylor et al., 2001). This resulted in more than 1 ortholog in zebrafish of 30% of mammalian genes. Thus, we must consider the possibility of a second *islet3* ortholog in zebrafish that might act redundantly with *islet3* with respect to eye development. This could be the reason why we are having difficulty obtaining a morphant phenotype. If our morpholino oligonucleotide is effectively blocking *islet3* mRNA translation but there remains another *islet3* ortholog that reacts redundantly with *islet3* with respect to eye development, the presence of functionally redundant protein encoded for by the second ortholog would explain out inability to observe a morphant phenotype.

To explore this possibility, we examined the puffer fish genome (*Fugu rubripes*) in search of a second *islet3* ortholog. This search did not yield a second ortholog. Similarly, a search of the currently available but incomplete genomic sequence of zebrafish did not return a second *islet3* ortholog. These findings suggest that there is not a second functionally
redundant islet3 ortholog in zebrafish. Though we have eliminated the possibility of redundancy with a second islet3 ortholog, there remains the possibility of redundancy with another related gene such as lhx2.

Alternatively, we must entertain the possibility that islet3 does not play a role in early eye development at all. This is in stark contrast to the results of the Kikuchi dominant/negative experiment which suggests islet3 plays an essential role in eye development, specifically in the evagination of the optic vesicles. How then, do we reconcile our islet3 knockdown results with the results of the Kikuchi dominant/negative experiment? There are two possibilities. First, LIM\text{isl3} may act as a promiscuous dominant/negative by antagonizing the activity of other Lhx proteins that play roles in eye development by binding and sequestering out their essential Ldb cofactors. Alternatively, it could act through an entirely Lhx-independent mechanism. LIM\text{isl3} could be involved in other protein interactions with GATA, bHLH, DEAF, CtIP, BRCA, or other factors to form and regulate the activity of transcription factor complexes that regulate eye development. In any case, LIM\text{isl3} could simply be mimicking the function of Lmo proteins, thereby resulting in a similar overexpression eyeless phenotype.

Currently, we have been unable to identify any other putative interactors with Lmo4 that might mediate its role in regulating eye
development. However, there is another candidate Lhx protein in the literature that could serve as a target for antagonism by Lmo4 in eye development. *Lhx2* knockout mice fail to develop eyes. Optic vesicles are specified in these embryos, but these vesicles fail to form optic cups. This strongly suggests that a *lhx2* gene identified in zebrafish could be a candidate target for antagonism by *lmo4*. To date, the identification of *lhx2* in zebrafish has not been published.

**Lmo4-Ldb interactions and structure-function relationships**

The literature in the field of LIM domain-containing proteins and their interactions shows us that there is evidence for specificity with respect to the preference of Ldb proteins to preferentially or exclusively bind to one of the tandem LIM domains in nuclear LIM proteins. Yeast 2-hybrid experiments conducted between Ldb1 and altered forms of Xlim1 (*Xenopus* Lhx protein) revealed that optimal binding required both LIM domains (Breen, 1998). The introduction of point mutations changing conserved cysteine residues to glycine into either the LIM A (A designates the N-terminal domain while B designates the C-terminal domain) or LIM B domains resulted in similar binding affinities, albeit at a reduced efficiency to the wild type Xlim-1 protein. However, when isolated LIM domains were tested, LIM A was
determined to have a much greater affinity for Ldb than LIM B alone. Functional analysis through site-directed mutagenesis of *apterous* (*Drosophila* Lhx) concluded that LIM B was essential for interaction with Chip (*Drosophila* Ldb) and function, whereas LIM A only bound weakly to Chip and is thus only required for full activity of the protein (Rincon-Limas et al., 2000). Additionally, *in vitro* experiments revealed that Ldb1 bound preferentially to LIM A of Lmo2 (Jurata and Gill, 1997). All of this data suggests at least some specificity regarding which LIM domain participates in interactions with Ldb proteins.

Additionally, in *Drosophila*, the *apterous* (Lhx) gene plays a role in both wing development and neuronal axon pathfinding. Experiments in which chimeric LIM-HD proteins were expressed by replacing the LIM domains of Apterous with the LIM domains from another Lhx protein revealed that LIM domains are interchangeable in wing development but not in neuronal axon pathfinding (O'Keefe et al., 1998). This suggests the existence of different interactions with the same LIM domain protein in different processes. Collectively, these results suggest that there is some specificity as to which LIM domain participates in interactions with Ldb proteins. However, there appears to be no general specificity as the preferred LIM domain varies between different nuclear LIM proteins.
In our analysis of Lmo4-Ldb interactions, we have determined that Lmo4 can interact with Ldb1, Ldb2, and Ldb3. The interaction is strongest with Ldb1 and weakest with Ldb3. Making point mutations in either LIM A or LIM B of full-length Lmo4 resulted in strengthening the ability of the mutated protein to interact with Ldb1. However, these point mutations did not significantly alter the strength of interactions with either Ldb2 or Ldb3. Similarly, a deletion mutants consisting of only LIM A demonstrated a stronger interaction with Ldb1 than wild type Lmo4. However, the interaction strengths between LIM A and Ldb2 or Ldb3 were comparable to wild type Lmo4. On the other hand, the deletion mutant consisting of only LIM B was able to interact with all three Ldb s more strongly than wild type Lmo4. However, the LIM B-Ldb1 interaction remained the strongest. Finally, mutating conserved aspartic acid residues in either LIM domain of Lmo4 into histidine did not result in different binding affinities. In summation, these results suggest that Lmo4 binds preferentially with Ldb1 and that the interaction can be mediated by both LIM domains of Lmo4. However, the interaction appears to be stronger with LIM B of Lmo4 than with LIM A.

Our results differ from results obtained by others (Deane et. Al, 2004) that suggest that full length Lmo4s interaction with Ldb1 is 1000 fold
stronger than that between individual LIM domains of Lmo4 with Ldb1. Furthermore, Deane et al. (2004) determined through a competition-based ELISA experiment that the interaction between Ldb1 and LIM A is several fold stronger than that between Ldb1 and LIM B.

Having identified and characterized Lmo4-Ldb interactions, our goal is to determine which interactions are important for the in vivo function of Lmo4. Specifically, is Ldb interaction sufficient for its function in eye development? This information will help us to differentiate between the lhx antagonism model and the multimeric transcription factor complex model as far as which mechanism Lmo4 might be utilizing to regulate eye development. Our lmo4 mutant gain of function analysis showed that overexpressing lmo4-C48G,C113G in which both LIM domains are mutated resulted in no statistical difference with WT embryos with respect to eye size. Likewise, there was no statistical difference in eye size when embryos were injected with RNA encoding LIM A. However, overexpressing LIMB of lmo4 did result in larvae with statistically smaller eyes compared to wild type. The small eye phenotype obtained with LIM B overexpression is not as severe as that obtained with full length lmo4 overexpression.

Thus, we can conclude that both LIM domains of Lmo4 are necessary to observe the robust eyeless overexpression phenotype. Interactions
mediated by both LIM domains must contribute to Lmo4s function in zebrafish eye development. We have determined by yeast two hybrid that both LIM domains can mediate Ldb interactions. However, our results suggest that LIM B is able to mediate stronger Ldb interactions than LIM A. LIM B by itself appears to be more active *in vivo* than LIM A. This suggests that interactions mediated by LIM B might play a more prevalent role than interactions mediated by LIM A. This correlates nicely with the fact that our results showed that LIM participates more strongly in Ldb interactions. Therefore, it appears that Lmo4 might regulate eye development predominantly through a mechanism involving Ldb interactions. However, we are unable to distinguish whether such a mechanism involves *Ihx* antagonism or the mediation and regulation of large multimeric transcription factor complexes.

**An analysis of residues important for Lmo-Ldb interactions**

The paper published by Deane et. al (2004) identified specific residues in human Lmo4 and human Ldb1 that are important for the interaction between the two proteins (Figure 9). These residues were also classified according to the types of interactions that they mediated. Their
findings can be used as a starting point for further analysis of residues important for Lmo-Ldb interactions.

A peptide sequence comparison of the LIM interaction domains from the four known zebrafish Ldb proteins (Figure 17) shows that the LIM interaction domain is highly conserved among all 4 L dbs. Ldb3 differs from the other L dbs in three respects: a serine at position 308 compared to a threonine for the other L dbs, a lysine at position 314 compared to a phenylalanine, and a valine at position 317 compared to a glutamic acid. The amino acid changes at positions 308 and 314 represent changes to similar amino acids in that they are polar and nonpolar respectively. However, the substitution at position 317 represents a dissimilar substitution. At that position, an acidic amino acid is replaced by a nonpolar valine. Interestingly, amino acid 317 was not determined to be a residue mediating an interaction between Ldb and Lmo by Deane et. al (2004).

| Human Ldb1-LID | DVMVVGEPPLMGGEFGDEDELITRL |
| Zfish Ldb1-LID | DVMVVGEPPLMGGEFGDEDELITRL |
| Zfish Ldb2-LID | DVMVVGEPPLMGGEFGDEDELITRL |
| Zfish Ldb3-LID | DVMVVGEPPLMGGEFGDEDELITRL |
| Zfish Ldb4-LID | DVMVVGEPPLMGGEFGDEDELITRL |

*Figure 17: Alignment of LIM interaction domains between human and zebrafish L dbs. Conservative substitutions are indicated in green whereas dissimilar substitutions are indicated in red.*
Our quantitative interaction assays showed that zebrafish Lmo4 interacts more strongly with Ldb1 than with Ldb2 or Ldb3. This is difficult to explain given the identity of the LIM interaction domains of Ldb1 and Ldb2 and the near identity of the LIM interaction domain of Ldb3 from that of Ldb1 and Ldb2. Perhaps there might be other residues that mediate important interactions or other specific interactions that were missed by Deane and colleagues. Additionally, the analysis undertaken by Deane et al. (2004) was limited to Lmo4's interaction with the LIM interaction domain only of Ldb1. It is possible that other regions of Ldb proteins might contribute to their ability to bind Lmo proteins.

A comparison of the LIM domains from human Lmo4, zebrafish Lmo4a and Lmo4b, and zebrafish Lmo2 shows that nearly half of the residue positions identified as mediating interaction between human Lmo4 and human Ldb1 are not 100% conserved in the three zebrafish Lmo protein sequences studied (Table 2). Though many of the differences represent conservative substitutions of similar amino acids, many residues are substituted by dissimilar amino acids. For example, the polar amino acid tyrosine at position 37 of human Lmo4 which is involved in hydrophobic interactions with a leucine at position 325 in human Ldb1 is replaced by a phenylalanine in zebrafish Lmo4b and a lysine in zebrafish Lmo2 which are
nonpolar and basic respectively. Similarly, the serine at position 63 of human Lmo4 that takes part in multiple hydrophobic interactions with human Ldb1 through residues glutamic acid at position 319, leucine at position 321 and isoleucine at position 322, is substituted by a phenylalanine in Lmo4a, a threonine in Lmo4b, and an arginine in Lmo2. This sequence analysis represents a starting point for future Lmo-Ldb interaction analyses. A comparison of the residues that have been identified as interaction mediators along with quantitative binding assays measuring the relative strength of different Lmo-Ldb interactions would provide further insight into the key residues important in determining the interaction strengths between Lmo members and Ldb members.
| a.a. position | 20 | 30 | 32 | 33 | 34 | 35 | 36 | 37 | 39 | 58 | 59 | 62 | 63 | 65 | 66 | 74 | 77 | 78 | 85 | 94 | 96 | 97 | 99 | 100 | 101 | 102 | 107 | 113 | 123 | 124 | 125 | 126 | 127 | 128 | 129 | 130 |
|--------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Zfish Lmo4a  | W  | I  | D  | R  | F  | L  | L  | V  | M  | L  | G  | E  | T  | F  | F  | Y  | T  | R  | V  | I  | G  | I  | A  | S  | E  | L  | V  | M  | R  | V  | F  | V  | P  | G  | D  | R  | F  | H  | V  |
| Zfish Lmo2   | S  | I  | D  | R  | F  | F  | L  | K  | I  | L  | G  | E  | R  | R  | V  | Y  | R  | V  | V  | L  | G  | I  | A  | F  | E  | M  | T  | M  | R  | V  | F  | C  | V  | G  | D  | R  | Y  | L  | L  |

Table 2: Comparison of the amino acids of 3 zebrafish Lmo proteins at positions corresponding to residues known to mediate human Lmo4-Ldb1 interactions.
The peptide sequences of human Lmo4, zebrafish Lmo4a, zebrafish Lmo4b, and zebrafish Lmo2 were aligned using ClustalW. This alignment only shows specific residues corresponding to amino acid positions in human Lmo4 that are known to be involved in human Lmo4-Ldb1 interactions. Residues that are not 100% conserved across the four proteins are highlighted in red.
In conclusion, we have initiated an analysis of the role of LIM domain containing proteins in zebrafish eye development. We have identified and characterized the lmo4 locus as negative regulator in the specification and/or evagination of the optic vesicles. We have begun to test our hypothesis that lmo4 might regulate eye development through antagonizing islet3 function. The inability to observe any eye developmental defect in islet3 morphant embryos together with the inability to rescue the lmo4 gain of function eye phenotype with concomitant islet3 overexpression suggests that islet3 may not be involved in zebrafish early eye development. Thus, other candidate LIM homeodomain targets for antagonism by lmo4 such as lhx2 must be identified and tested. Furthermore, we have initiated a study of the structure and function of Lmo-Ldb interactions using Lmo4 as a model. We have determined that Lmo4 interacts preferentially with Ldb1 to Ldb2 and Ldb3. Either LIM A or LIM B of Lmo4 is able to mediate Ldb interactions. However, our results suggest that LIM B is able to mediate a stronger interaction with Ldbs that LIM A. This correlates with in vivo functional analyses with show that overexpressing LIM B results in a mild small eye
phenotype whereas overexpressing \textit{LIM A} does not produce a statistically significant eye phenotype.
APPENDIX A

Isl3 HD-Ldb DD Fusion

The inability to rescue the *lmo4* overexpression eye phenotype via *islet3* overexpression suggests that Islet3 may not be a target for antagonism by Lmo4 at least in the process of eye development. In order to further test these findings, we have designed fusion constructs (Milan and Cohen, 1999). We have made a fusion construct in which the DNA-binding homeodomain of *islet-3* is fused to the dimerization domain of the *ldb* (Figure 18). The fusion protein encoded by these constructs should not be dependent on Ldb binding for activation. They should be able to dimerize themselves and be active. Therefore, embryos injected with mRNA encoding these Islet3\textsuperscript{HD}-Ldb\textsuperscript{DD} fusion proteins should be unaffected by manipulated levels of *lmo4*. This experiment will tell us whether the transcriptional activity of Islet3 is sufficient for eye development.
Figure 18: Schematic of Islet3<sup>HD</sup>-Ldb<sup>DD</sup> fusion experiment.
A. Normal association of Islet3 (green) with Ldb (blue) allows the formation of a functional tetrameric transcription factor complex.
B. The Islet3<sup>HD</sup>-Ldb<sup>DD</sup> fusion protein will fuse the dimerization domain (DD) of Ldb1 with the homeodomain (HD) of Islet3, thereby releasing it from dependence on Ldb binding for functionalization. LID-LIM interaction domain.

If Islet3 positively regulates eye development and serves as a target for antagonism by Lmo4 with respect to eye development, we would expect this constitutively active fusion protein to cause a lmo4 loss of function phenotype—an expanded retina. Additionally exogenous Lmo4 should not be able to affect the Islet3<sup>HD</sup>-Ldb<sup>DD</sup> fusion protein phenotype.

However, when we inject RNA encoding the Islet3<sup>HD</sup>-Ldb<sup>DD</sup> fusion, embryos become severely malformed and dorsalized. The ectopic expression of the Islet3<sup>HD</sup>-Ldb<sup>DD</sup> may be resulting in a neomorphic phenotype. Therefore, we may have to restrict expression of the fusion protein to the eye field by using an eye-specific promoter to be able to accurately assess eye
phenotype devoid of other morphologic abnormalities that may hinder our analysis. To this effect, an eye-specific promoter was obtained from the Jamrich lab consisting of the zebrafish \textit{rx1} promoter. This promoter has been shown by the Jamrich lab to drive eye-specific expression of transgenes in a mosaic manner (data unpublished).
Cloning zebrafish *rlim*

Finally, RLIM is a RING finger LIM domain-binding protein that has been described in mice, chick, and human as an ubiquitin protein ligase. Functional studies of RLIM have shown it to be a corepressor of LIM domain-containing proteins and Ldb5s by binding them and targeting them for degradation through ubiquitination (Ostendorff et al., 2002). Thus, any RLIM discovered in zebrafish would be a candidate interactor to Lmo4 as well as a potential regulator of Lmo4 protein accumulation.

We have initiated efforts towards cloning zebrafish *rlim*. The mouse *rlim* sequence was blasted against zebrafish genomic shotgun sequence. This produced a ~300 bp match with an e-value of 1e-34. This partial sequence has high homology with the mouse RLIM protein from residues 498 to 593, which includes the RING finger consisting of residues 546 to 586. This fragment was cloned into pBS using primers DJ46 and DJ47 and the restriction enzyme sites EcoR1 and Spe1 for probe making. Preliminary *in situ* hybridization experiments reveal a high level of background making it difficult to discern specific from non-specific staining. An accurate expression pattern for zebrafish *rlim* can not be obtained using our short 300
probe. The full length cDNA will have to be cloned for further expression analysis. Additionally, primers were designed for 5’ and 3’ RACE PCR to clone the full length \textit{rilm} cDNA.
Works Cited


