Role of Zebrafish Lbx2 in Embryonic Lateral Line Development

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Abstract

Background: The zebrafish ladybird homeobox homologous gene 2 (lbx2) has been suggested to play a key role in the regulation of hypaxial myogenic precursor cell migration. Unlike their lbx counterparts in mammals, the function of teleost lbx genes beyond myogenesis during embryonic development remains unexplored.

Principal Findings: Abrogation of lbx2 function using a specific independent morpholino oligonucleotide (MO) or truncated lbx2 mRNA with an engrailed domain deletion (lbx2eh) resulted in defective formation of the zebrafish posterior lateral line (PLL). Migration of the PLL primordium was altered and accompanied by increased cell death in the primordium of lbx2-MO-injected embryos. A decreased number of muscle pioneer cells and impaired expression pattern of sdf1a in the horizontal myoseptum was observed in lbx2 morphants.

Significance: Injection of lbx2 MO or lbx2eh mRNA resulted in defective PPL formation and altered sdf1a expression, confirming an important function for lbx2 in sdf1a-dependent migration. In addition, the disassociation of PPL nerve extension with PLL primordial migration in some lbx2 morphants suggests that pathfinding of the PLL primordium and the lateral line nerve may be regulated independently.

Introduction

The zebrafish lateral line system consists of a set of neuromasts, important mechanosensory organs which detect hydrodynamic variations and water currents, and their underlying neurons regularly arrayed along the surface of the head and body [1,2,3,4,5]. Neuromasts are composed of hair cells (HCs) and their characteristic surrounding cells, mainly mantle cells and supporting cells. HCs in the zebrafish lateral line system have a similar morphology and function to human HCs, and due to the external location, the zebrafish lateral line system has evolved into a strong model for investigation of HC toxicity, regeneration and protection, as well as screening for drugs to cure diseases associated with hearing loss in humans [6,7,8].

Development of the lateral line system in zebrafish has been studied at the embryonic stages, including control of the directional migration of the lateral line primordium, deposition and differentiation of neuromasts in the posterior lateral line (PLL) and mechanisms of HC polarity and regeneration. Until recently, several widespread signaling pathways including sdf1a-cxcr4b/cxcr7, Fgf, Notch and Wnt signaling have been suggested to synergistically function in the formation and maintenance of the lateral line system [9,10,11,12,13]. Many genes which are expressed in the migrating primordium and are putatively thought to be responsible for embryonic lateral line development are now being characterized [14]; however, genes which are expressed at undetectable levels in the PLL system, but which are able to affect the formation of PLL are largely elusive.

The zebrafish gene, lbx2, is one of several vertebrate counterparts of the ladybird family of homeobox genes in Drosophila, and has been proven to regulate myofibril formation and fin bud development [15]. Lbx gene family members are characterized by a N-terminal engrailed repressor domain. Amongst the vertebrates, mouse lbx1 and lbx2 were identified in 1999 [16] have been widely investigated. Marine lbx1 has been reported to be necessary for myogenesis [17,18,19,20,21], neuronal development [22,23,24,25,26,27,28,29] and neural crest-derived tissues [30]. Although apparent abnormalities are not detected in lbx2-null mice, lbx2 may possibly be involved in ovarian development and folliculogenesis [31]. Further studies on the expression patterns and developmental roles of existing members of the lbx gene family are required to expand our knowledge of the evolution of these genes in vertebrates [32].

In this study, analysis of the phenotypes of lbx2 morphants demonstrated that depletion of lbx2 leads to PLL malformations in zebrafish. The similar PLL defects observed in both lbx2 morphants and embryos injected with truncated lbx2 mRNA with an engrailed domain deletion (lbx2eh) suggest a functional role for lbx2 in development of the PLL. Integrity of the supporting cell and HC population in deposited neuromasts remained intact in...
lbx2 morphants. However, an impaired expression pattern of sdf1a in the horizontal myoseptum was observed in lbx2 morphants, as well as a defective migration pattern and increased cell death in the migrating PLL primordium. This study adds to the existing knowledge of the role of zebrafish lbx2, and deepens the understanding of lateral line development.

Results

Abnormal pattern of posterior lateral line neuromast deposition in lbx2 morphants

To study the function of lbx2 in early zebrafish development, we significantly reduced lbx2 protein expression by employing a lbx2 specific morpholino (MO), which targets the AUG start codon of lbx2 mRNA to block translation. Firstly, we confirmed the specificity and efficiency of the lbx2 MO. In order to test the efficiency of lbx2 translational inhibition in vivo, we constructed an lbx2-EGFP reporter fusion protein construct, containing 60 bp of the 5′-UTR and the coding region for the first 66 amino acids of zebrafish lbx2 fused to the N-terminus of EGFP and a SV40 polyadenylation site, in the expression vector pEGFP-N1 (see Materials and Methods and Figure S1 for details). Transcription of lbx2-EGFP can be constitutively driven by the active human cytomegalovirus (CMV) promoter in embryos (Fig. S1A); therefore, we could detect translation of lbx2-EGFP by visualizing GFP fluorescence in embryos injected with linearized plasmid. When the lbx2-EGFP construct was co-injected with lbx2 MO into zebrafish embryos, diminished GFP fluorescence was observed, indicating that translation of lbx2-EGFP can be effectively blocked by lbx2 MO (Fig. S1B and S1C). Using an antibody against zebrafish lbx2 which was developed in our laboratory, we detected lower lbx2 protein expression in lbx2 morphants using Western blot analysis (Fig. S1D). Zebrafish lbx2 has been suggested to play an essential role in myogenesis in the pectoral fin bud [15]. We observed an absence of myoD-positive cells in the pectoral fin bud region of most lbx2 morphants, which could be rescued by co-injection of lbx2 mRNA with the lbx2 MO (Fig. S1E). In order to ensure that our experimental observations on the specific function of lbx2 were accurate, we synthesized a mutated form of lbx2 mRNA lacking the engrailed repressor domain, named lbx2mRNA, for complementary analyses. The function of the truncated mRNA was indicated by depletion of myoD expression in the pectoral fin bud of embryos injected with lbx2mRNA (Fig. S1F–I). Taken together, lbx2 MO and lbx2mRNA provide specific and efficient tools for functional studies of lbx2.

We injected zebrafish embryos at the one-to-two cell stage with lbx2-MO and found that most lbx2 morphants exhibited circling swimming behavior at the larval stage. Similar to lbx2 morphants, lbx2mRNA injected embryos also showed abnormal swimming behavior. Previous work has suggested that the mechanosensory lateral line system controls various types of swimming behavior in zebrafish [33]; therefore, we dissected the phenotype in order to analyze lateral line development in lbx2 morphants.

Analysis of lateral line system development was conducted using the following experiments. Firstly, Claudin b (cldnb), a gene specifically expressed in PLL primordium and neuromast cells, was employed as a marker to observe the number and position of PLL neuromasts. The pattern of cldnb expression was examined in embryos at 48 hpf, at which stage neuromast sets have been deposited along the horizontal myoseptum to the tip of the tail during embryonic PLL primordium migration. As shown in Figure 1, 100% (57/57) of the embryos injected with control MO displayed the normal pattern of 5–7 cldnb-positive neuromasts; however, most lbx2 MO injected embryos (39/41) showed decreased numbers of labeled neuromasts (average of three), and 15% (6/41) of the morphants lost all of the neuromasts (Fig. 1A–1D).

4-(4-diethylaminostyryl)-N-methylpyridinium iodide (DiAsp) can be incorporated by terminally differentiated HCs in lateral line neuromasts; therefore, DiAsp staining is an effective method for the detection of functional mature HCs [34]. At 48 hpf, neuromast HCs in control embryos were clearly stained with DiAsp, while faint DiAsp staining and decreased numbers of neuromasts were observed in lbx2 morphants (Fig. 1E and 1F). Presence of the engrailed domain in lbx2 has been suggested to be critical for proper gene functioning [15]. In order to verify that the PLL defect observed in lbx2-deficient embryos was caused by functional depletion of lbx2, lbx2mRNA was injected into SqET4 embryos. SqET4 is a recently developed transgenic zebrafish line expressing high levels of GFP in HC progenitors in the PLL [35]. An abnormal pattern of PLL neuromast deposition was observed in SqET4 embryos injected with lbx2mRNA (Fig. 1G and 1H), similar to the PLL defects observed in lbx2 morphants (Fig. 1B and 1F). These results suggest that impaired lbx2 function specifically reduces the number of neuromasts and induces malformations in the PLL.

The integrity of progenitor cells in deposited neuromasts and organization of the PLL primordium in lbx2 morphants

It has been shown that notch3 is expressed in support cells of PLL neuromasts [36]. Given the significant change in the neuromast number of lbx2 morphants, we studied the support cell population in deposited neuromasts after lbx2 depletion at 48 hpf. The number of notch3-expressing cells in the deposited neuromasts of lbx2 morphants was comparable to control embryos (Fig. 2A and 2B). Using SqET10, a transgenic zebrafish expressing GFP in PLL supporting cells and nerves [35], and SqET4 embryos, we observed a similar pattern of GFP expression in the HCs and supporting cells of deposited neuromasts in lbx2 morphants and control embryos (Fig. S2A and S2B). This data suggested that the supporting cells and HCs of deposited neuromasts were largely unaffected in lbx2 morphants, indicating that lbx2 is not essential for the survival and differentiation of supporting cells and HCs once neuromasts have been correctly deposited.

Initial formation of the PLL primordium requires cellular organization into protoneuromast rosettes. Migration of the PLL primordium begins when the leading two-to-three rosettes form; and then deposition onset occurs, followed by formation of a fourth rosette. Fgf ligands and cxcr4b are always expressed in the leading zone of the newly formed two or three rosettes, and control organization and polarity of the migrating PLL primordium [34]. Therefore, we performed whole-mount in-situ hybridization using antisense probes for fgg10 and cxcr4b, and observed no obvious difference in the organization of newly deposited protoneuromast rosettes in lbx2 morphants and controls (Fig. 2C–2F). These results reveal that lbx2 is not involved in cellular organization or polarity of the PLL primordium. We also evaluated several other key components of pathways participating in PLL formation. Lef1 are p25 are targets of wnt/beta-catenin and Fgf signaling, respectively; and cxcr7 is a sdf1a receptor which is expressed in the posterior regions of the migrating PLL primordium. There was no appreciable difference in the expression of lef1, p25 and cxcr7 in the migrating PLL primordia of lbx2 morphants and control embryos (Fig. 2G–2L), indicating that although lbx2 is essential for performance of lateral line system functions in response to dynamic external stimuli (Fig. 1 and 2), lbx2 is not essential for
regular signal integrity and cell differentiation in the PLL primordium.

The lateral line nerve is not affected in \textit{lbx2} morphants

The PLL placode also generates PLL sensory neurons during primordium migration. Sensory growth cones remain associated with the migrating PLL primordium and form the PLL nerve. Recently, migration and development of the PLL nerve were shown to be stalled or misrouted during the malformation of PLL neuromasts when \textit{sdf1a-cxcr4b} signaling is disrupted \cite{36,37}. In order to assess the possible function of \textit{lbx2} in PLL nerve development, anti-acetylated-\textalpha-tubulin antibody was used to immunohistochemically label axons of the PLL or the lateral line nerve ganglion in \textit{lbx2} morphants \cite{36}. Unexpectedly, no obvious abnormalities were observed in the PLL nerves of most \textit{lbx2} morphants at 48 hpf (Fig. 3A and 3B). By injecting the \textit{lbx2} MO, we found that the PLL axon could continue to extend in larva, even if neuromast deposition had stalled (Fig. 3C and 3D). The results suggest that PLL axons can be guided independently from PLL primordial migration via different molecular signaling systems.

**Impaired migration and increased cell death in the PLL primordium of \textit{lbx2} morphants**

We monitored the process of PLL primordium migration at several developmental stages from 30 to 48 hpf in control and \textit{lbx2}-injected embryos by whole mount \textit{in situ} hybridization using a probe for \textit{atoh1a}, a marker of HCs in the migrating PLL primordium \cite{38}. The leading edge of primI can be determined by \textit{atoh1a} expressing cells. Ten embryos from the \textit{lbx2}-MO injected group and control-MO injected group were examined at 36, 40 and 48 hpf. A significant reduction in the speed of primordium migration was observed in nearly all of the \textit{lbx2} morphants tested (Fig. 4A–4G). In the most defective morphants, neuromasts were deposited in the anterior part of the migratory route (data not shown). In most of the moderately affected morphants, neuromasts were deposited in inappropriate locations as the primordium extended posteriorly at a reduced speed. Notably, similarly to other PLL primordium migration defects observed in a variety of zebrafish mutants and morphants \cite{37}, nearly all of the \textit{lbx2} morphants possessed a correctly deposited first neuromast. To assess whether primordium migration defects could lead to increased cell death, we analyzed cell death in both control-MO...
and lbx2-MO-injected embryos at 36 hpf using whole-mount TUNEL staining. As shown in Figure 4H and 4I, the lbx2 morphant primordium and deposited neuromasts (visualized as light violet dot clusters by DAPI staining) contained significantly higher numbers of TUNEL-positive cells (labeled as red dots) compared to control embryos. This suggests that stalled PLL primordium and/or misdeposited neuromasts in lbx2 morphants may undergo apoptosis. Thus, overall disorganization of the PLL neuromasts in lbx2 morphants could primarily be due to impaired primordium migration caused by loss of lbx2 function.

Lbx2 deficiency in zebrafish affects expression of sdf1a

Sdf1a is the main chemokine which drives correct migration of the PLL primordium via binding to cxcr4b. By 16 hpf, sdf1a is expressed in the posterior lateral mesoderm and adaxial cells. Sdf1a is synthesized by muscle pioneer cells and secreted into the horizontal myoseptum, and binds to cxcr4b in PLL primordium cells, thus guiding PLL migration [36]. Overlapping expression domains of zebrafish lbx2 and sdf1a are present in the posterior lateral mesoderm at the tail bud stage (Fig. 5A and 5B) and in adaxial cells at the 10 somite stage (Fig. 5C). We have also observed partial co-localization of lbx2 and sdf1a expression in adaxial cells and cells of the horizontal myoseptum at later developmental stages, as previously described [15,36]. The existence of overlapping expression domains prompted a hypothesis of crosstalk between lbx2 and sdf1a. We examined the expression pattern of sdf1a in lbx2-injected embryos at 24 hpf. In contrast to control embryos, most lbx2 morphants had a weak and discontinuous pattern of sdf1a expression just before the PLL began to migrate (Fig. 6A–D). This defective sdf1a expression pattern could be replicated by injection of lbx2eh-mRNA into zebrafish embryos (Fig. 6E and 6F), and was reminiscent of the fragmentation which occurs when the PLL primordium migrates to the tip of the tail.

Impaired sdf1a expression along the horizontal myoseptum could be the result of damage to the anatomical integrity of the horizontal myoseptum [36], or loss of sdf1a-expressing pioneer cells. Using an antisense probe to tenascin C (a marker of the horizontal myoseptum [39,40]), we measured the anatomical integrity of the zebrafish horizontal myoseptum. As shown in Figure 6G and 6H, the myoseptum was not disturbed in lbx2 morphants. However, after a careful examination of muscle pioneer cells, using riboprobe hybridization of the muscle pioneer cell marker eng2a or 4D9 antibody staining [41], we found that fewer muscle pioneer cells formed in lbx2 morphants (Fig. 6I–6N). These results suggest that the abnormal pattern of sdf1a expression in lbx2 morphants may possibly be due to interference with the differentiation of sdf1a-expressing cells in the horizontal myoseptum after abrogation of lbx2. Although expression of lbx2 was never observed in the PLL primordium, our results suggest that...
indicates the stalled primordium. Embryos used in the assay were at 48
black arrows show the deposited neuromasts and the black asterisk
(D), but not in control embryos (C). White arrows indicate the PLL nerve,
revealing disassociation of the primodium and nerve in
Discussion
ainenesis of zebrafish; however, the functions of
lbx2 morphants. (D) Immunohistochemical detection of the PLL nerve using an anti-
acetylated α-tubulin antibody, indicating that the PLL nerve grew
correctly in both control embryos (A) and lbx2 morphants (B). (C, D)
immunohistochemical detection of the PLL nerve using an anti-
acetylated alpha tubulin antibody to label PLL neuromasts and in situ
hybridization using a cldnb antisense probe to label the primordia,
revealing disassociation of the primodium and nerve in lbx2 morphants
(D), but not in control embryos (C). White arrows indicate the PLL nerve,
black arrows show the deposited neuromasts and the black asterisk
indicates the stalled primordium. Embryos used in the assay were at 48 hpf stage.
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lbx2 may interfere with the stereotypical pathway of the migrating
PLL primordium via dysregulated secretion of sdf1a along the
myoseptum.

Discussion
Ladybird homeobox (Lbx) genes are a group of homeodomain-
containing genes related to the Drosophila ladybird genes [42].
During post-gastrulation limb and fin development in zebrafish,
lbx1 and lbx2 display a conserved expression pattern in a subset of
hypaxial myoblasts. Before hypaxial myoblasts migrate to the
limb/fins buds, they transition from epithelial to mesenchymal
cells in the dermomyotome. Loss of lbx1 and lbx2 in animal models
results in a lack of limb/fin musculature attributed to muscle
progenitor cells migration defects [15,18,43,44,45]. Knockout
studies in mice suggest that lbx1 is involved in interpreting the
signals which guide appendicular muscle precursor migration
[18,43,44], and lbx2 is involved in development of the pectoral fin
bud muscle in zebrafish [15]. The expression pattern of lbx2 has
been characterized in zebrafish; however, the functions of lbx2 in
differential neural tissue development during early embryogenesis
remain largely unknown.

Previous studies have suggested that a number of cellular or
molecular relationships between muscle development and lateral
line development exist [41,46,47]. These studies have mainly
focused on genes (such as met and pax6), whose transcripts are
present in both muscle tissue and the lateral line system. However,
this study reveals a novel function for lbx2 in the control of PLL
migration via the modulation of sdf1a expression. The.
expression of lbx2 and sdf1a in the early embryonic stages has
already been observed. This study demonstrates that lbx2 is
involved in maintenance of the precise expression pattern of sdf1a
in the horizontal myoseptum. Thus, the loss of lbx2 function, due
to injection of lbx2 MO or lbx2-wt mRNA, lead to abnormalities in
the expression pattern of sdf1a and affected the ability of sdf1a to
guide migration of the PLL primordium. As lbx2 contains an
engrailed domain, it is normally considered to be a transcriptional
repressor. Our results suggest that the putative repressor domain
of zebrafish lbx2 is required to maintain the correct pattern of sdf1a
expression in the horizontal myoseptum. Therefore, sdf1a is
unlikely to be a direct repression target of lbx2. Previous studies
have suggested zebrafish lbx2 plays a role in muscle precursor
differentiation and myofibril formation [15], and we observed
decreased numbers of eng2a or 4D9 positive muscle pioneer cells in
lbx2 morphants (Fig. 6), demonstrating that zebrafish lbx2 plays an
important role in the regulation of sdf1a-expressing muscle pioneer
cell differentiation. This study strongly suggests that regulation of
lateral line primordium migration by the sdf1a-cxcr4b-signaling
cascade requires expression of lbx2, even though expression of lbx2
cannot be detected in the lateral line.

In parallel with our observation that depletion of lbx2 leads to
defective migration of the PLL primordium (Fig. 1), the neuromasts
deposited along the migration path remained largely intact (Fig. 2
and Fig. S2). The overall malformation of the PLL in lbx2
morphants could result from elevated cell death in misplaced or
stalled neuromasts at aberrant locations along the migration route
(Fig. 4). It has been suggested that both the PLL primordium and
axons are guided by sdf1a-cxcr4b signaling [36,37] or signals
present in the migrating PLL primordia [48]. We extended our
studies to the PLL nerve in lbx2 morphants. Interestingly, the
growth and extension of the PLL nerve in lbx2 morphants
appeared normal, even in the presence of a high level of
misposition or stalling of the primordia (Fig. 3C-3D). This
indicates that extension of PLL axons is guided independently of
PLL primordium migration, suggesting that formation of the PLL
nerve may not ultimately depend on the sdf1a-cxcr4b system or
movement of the PLL primordium. Although we still cannot
define the exact cause of the abnormal swimming behavior
observed in lbx2 morphants, it is possible that sensory organ
defects or malformation of the pectoral fin, rather than defects in
the PLL, may lead to abnormal swimming behavior.

In conclusion, this study describes a previously unrecognized
role for the involvement of lbx2 in zebrafish PLL formation, via a
regulatory function in muscle development. Comparative analysis
and molecular dissection of the migrating PLL could provide us
with more information about the early history of animal evolution,
as three lbx genes, lbx1a, lbx1b and lbx2, are present in the genome
of teleost fish such as zebrafish [15,32].

Materials and Methods
Zebrafish maintenance
Wild-type zebrafish, and the SqET4 and SqET10 trap lines
specifically expressing EGFP in the lateral line (gifts of Prof. V.
Korzh at the Institute of Molecular and Cell Biology, Singapore),
were maintained as previously described [49]. Embryos were

Figure 3. Disassociation of the PLL primordium and PLL nerve in
lbx2 morphants. (A, B) Labeling of the PLL nerve using an anti-
acetylated α-tubulin antibody, indicating that the PLL nerve grew
correctly in both control embryos (A) and lbx2 morphants (B). (C, D)
immunohistochemical detection of the PLL nerve using an anti-
acetylated alpha tubulin antibody to label PLL neuromasts and in situ
hybridization using a cldnb antisense probe to label the primordia,
revealing disassociation of the primodium and nerve in lbx2 morphants
(D), but not in control embryos (C). White arrows indicate the PLL nerve,
black arrows show the deposited neuromasts and the black asterisk
indicates the stalled primordium. Embryos used in the assay were at 48 hpf stage.
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collected by natural matings of zebrafish adults and incubated in egg water at 28.5°C [49]. Embryos at different developmental stages were staged by hours post fertilization (hpf) [50].

Whole mount in situ hybridization and antibody staining
For whole mount in situ hybridization, embryos older than 48 hpf were incubated in egg water containing 0.003% 1-phenyl-2-thiourea (PTU, Sigma, St. Louis, MO, USA) from 12 hpf onwards to prevent pigmentation. Embryos reaching the desired developmental stages were fixed in 4% PFA/PBS overnight at 4°C, dehydrated in 100% methanol and stored at −20°C before use. Whole-mount in-situ hybridization procedures (WISH) and double in-situ hybridization were performed as previously described [51]. Antisense probes for cldnb, fgf10, sdf1a, cxcr4b, cxcr7, tenascin C, atoh1a, lbx2, lef1, eng2a, pea3 and notch3 were synthesized using T7 or SP6 RNA polymerases and labeled with digoxigenin-UTP or fluorescein-UTP (Roche, Mannheim, Germany). The detailed whole-mount immunohistochemistry staining procedure has previously been described [52]. The antibody against acetylated α-tubulin (Santa Cruz Biotechnology, CA, USA) and 4D9 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) were used at 1:200 or 1:100 dilution. The primary rabbit polyclonal antibody against zebrafish lbx2 used for Western blotting was developed in our laboratory, purified and used at 1:50 dilution. For double staining using the cldnb antisense probe and anti-acetylated α-tubulin antibody, in situ hybridization was followed by immunohistochemical staining.

In-vitro mRNA synthesis
Wild-type lbx2 cDNA or truncated lbx2 cDNA lacking the repressor engrailed domain [15] were cloned into the pSP64-T vector (Promega, Madison, WI, USA). Capped lbx2 and lbx2eh mRNAs were transcribed from the linearized plasmids using the mMachine in-vitro transcription kit (SP6; Ambion, Austin, TX, USA) according to the manufacturer’s instructions.

Embryo micro-injection
The sequence of the lbx2 translation-blocking morpholino oligonucleotide (MO) was 5’-ctactggagtcgattgcc-3’ (ATG
positive muscle pioneer cells in control embryos (I) and (K) and J (L), showing decreased numbers of (J) at 30 hpf. (K, L) Magnified views of the expression patterns shown in I doi:10.1371/journal.pone.0029515.g006

All images are lateral views. White arrows indicate the hybridization or immunostaining signals. Embryos (G) and in a defective complementary sequence underlined). Lbx2 morphants at 24 hpf (F). (G, H) Similar expression pattern of lbx2, a marker of the horizontal myoseptum, in control embryos (E), injection of lbx2-mRNA resulted in a defective lbx2 expression at 24 hpf (F). (G, H) Similar expression pattern of tenascin C, a marker of the horizontal myoseptum, in control embryos (G) and lbx2 morphants (H) at 30 hpf. (I, J) Analysis of eng2a positive muscle pioneer cells in control embryos (I) and lbx2 morphants (J) at 30 hpf. (K, L) Magnified views of the expression patterns shown in I (K) and J (L), showing decreased numbers of eng2a positive muscle pioneer cells in lbx2 morphants (L) compared to control embryos (K). (M, N) Compared with control embryos (M), the numbers of 4D9 positive muscle pioneer cells was slightly reduced in lbx2 morphants (N) at 30 hpf. White arrows indicate the hybridization or immunostaining signals. All images are lateral views. doi:10.1371/journal.pone.0029515.g006

complementary sequence underlined). lbx2-MO (0.5 mM), standard-MO (0.5 mM) or 50 ng/ul lbx2 wild-type or truncated mRNA were dissolved in sterile double-distilled water containing phenol red and injected into one-two-cell stage zebrafish embryos using a Harvard micro-injector (Harvard Apparatus, Holliston, MA, USA). The effectiveness of zebrafish lbx2 MO translational inhibition was tested by detection of lbx2-EGFP green fluorescent fusion protein in vivo, which is described in more detail in Supplementary Figure S1.

DiAsp staining
DiAsp (4-Di-2-Asp, Sigma D3418) was dissolved in double distilled water at 500 mM and stored at 4°C. To label the neuromast hair cells, live 48 hpf morphants and control embryos were incubated in egg water containing 5 mM DiAsp for exactly 5 min, rinsed several times with fresh egg water for 5 min as previously described [53], and visualized using fluorescence microscopy.

TUNEL staining and measurement of PLL length
The TUNEL (Terminal dUTP Nick-End-Labeling) assay to detect cell death in zebrafish embryos was carried out as previously described [54], then after several washes in PBST, the embryos were transferred to DAPI solution to visualize the nuclei of the PLL primordium.

Ten randomly selected embryos (over 48 hpf) from morphants and controls were analyzed. The distance between the first neuromast and the end of primordium relative to the length of the body axis curvature was measured using ImageJ software (NIH). Significance analysis was conducted via Student’s t-test in Excel.

Supporting Information
Figure S1 Efficiency of the lbx2 morpholin. (A) A test lbx2-EGFP construct was created containing 60 bp of the 5’ UTR and the first 66 amino acid coding sequence of lbx2 cDNA fused to the N-terminus of EGFP, driven by the CMV promoter. The sequence of lbx2 MO is complementary to the 1–24 bp region of zebrafish lbx2 cDNA. (B) Live embryos at the 50% epiboly stage. Embryos co-injected with 25 ng lbx2-EGFP DNA and 5 ng control MO expressed green fluorescent fusion protein (left), which was inhibited by co-injection of 2 ng lbx2 MO (right). (C) Translation of lbx2-EGFP in live embryos was inhibited by co-injection of lbx2 MO. (D) The lbx2 protein level in lbx2-MO-injected embryos was drastically lower than control MO injected embryos at 30 hpf (E). Absence of MyoD expression in the pectoral fin bud of lbx2 morphants at 48 hpf, which could be rescued by co-injection of lbx2 mRNA. Arrowhead indicates MyoD expression in the pectoral fin bud area. (F–I) Injection of lbx2-mRNA dramatically inhibited MyoD expression in pectoral fin muscle precursors at 30 hpf (G) and 36 hpf (I), compared to gfp mRNA-injected control embryos (F, H). (TIF)

Figure S2 PLL cells in the newly deposited neuromasts of lbx2 morphants appear normal. (A–B) Hair cells of PLL neuromasts labeled with GFP in the SqET4 transgenic zebrafish line. The pattern and numbers of PLL hair cells in newly deposited neuromasts was similar in control embryos (A) and lbx2 morphants (B) at 48 hpf. (C, D) Fluorescence images of SqET10 embryos indicating that the supporting cells and lateral line nerve in newly deposited neuromasts of embryos injected with control MO (C) or lbx2 MO (D) are similar at 48 hpf. The white arrowhead indicates HGs in deposited neuromasts. (TIF)

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Author Contributions
Principal investigator, design, and preparation of the manuscript: ZY. Lbx2 morpholin designed and assessment experiment: QL. Conducted most of the experiments: XC. Designed experiment and some data collection: JH.
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