A Hox gene controls lateral line cell migration by regulating chemokine receptor expression downstream of Wnt signaling

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The posterior lateral line primordium in zebrafish provides an amenable model to study mechanisms of collective cell migration. The directed migration of the cell cluster along the path of Sdf1a chemokine requires two receptors, Cxcr4b and Cxcr7b, which are expressed in the leading and trailing part of the primordium, respectively. The polarized expression of receptors is regulated by Wnt signaling, but downstream players mediating this control remain to be found. Here, we show that the Hox homeobox gene Hoxb8a is a critical component that acts downstream of the Wnt pathway to coordinate the expression of both chemokine receptors. We find that Hoxb8a is expressed in the leading part of the primordium and is required for the correct speed and extent of migration. Hoxb8a expression is dependent upon Wnt activity and needed both for *cxcr4b* expression and to repress and thus restrict *cxcr7b* expression to the trailing zone of the primordium. In the absence of Wnt activity, overexpressed Hoxb8a is able to repress *cxcr7b* but not up-regulate *cxcr4b* expression. Together with results from expressing dominant activator and repressor constructs, these findings suggest that Hoxb8a is induced by and cooperates with Wnt signaling to up-regulate *cxcr4b*, and acts through multiple mechanisms to repress *cxcr7b* expression.

directional cell migration | Wnt-Hox-chemokine receptor pathway

During collective migration, cells remain in contact with each other and move as a coherent group with distinct front and rear polarity (1). The formation of the posterior lateral line in zebrafish is an amenable model for studying the molecular control of collective movement. The posterior lateral line arises from a group of cells, the primordium, that migrates along the anteroposterior axis of the embryo and distributes the future sensory organs (neuromasts) at regular intervals (2). This directional migration requires the Sdf1a/Cxcl12a chemokine, which is present in the horizontal myoseptum of somites along the path, and its receptor Cxcr4b, which is expressed in the primordium (3–5). Migration also requires Cxcr7b, which is expressed in the trailing part of the primordium and deposited cells, and has been proposed to provide directionality by acting as a sink for Sdf1a (6–8).

In the leading part of the primordium, activation of the canonical Wnt pathway regulates multiple aspects of posterior lateral line development, including proliferation, proneuromast formation, and primordium migration (9–13). By regulating the expression of specific Fibroblast Growth Factors (Fgfs) and the Fgf inhibitor Sef1, Wnt signaling induces and restricts Fgf signaling to the trailing zone. In turn, the Fgf pathway triggers epithelial morphogenesis of the neuromasts (14–17) and restricts Wnt activity to the leading part. This double-feedback interaction is essential for the asymmetric expression of chemokine receptors required for directional migration (13, 16). The regulation of chemokine receptor expression by Wnt signaling is in part mediated by Lef1, which acts in parallel with unidentified components (9). This raises the question as to whether a transcription factor(s) up-regulated by Wnt signaling mediates the regulation of chemokine receptor expression.

Hox genes encode transcription factors best known for their conserved role in axial patterning during development (18–20).

They contain a conserved homeodomain that mediates binding to DNA (21) and share similar DNA binding preferences in vitro, with their in vivo specificity thought to result from interactions with cofactors, such as three amino acid loop extension (TALE) proteins of the PBX and MEIS families (22, 23). In addition, Hox genes have been implicated in the regulation of cell migration, for example of subpopulations of neurons along the anteroposterior axis of the hindbrain (24–26). In *Caenorhabditis elegans,* a Hox gene is required for the migration of specific neuroblasts under the control of the Wnt pathway (27). However, functions of Hox genes in cell migration remain largely unexplored, and it is therefore important to identify downstream target genes that mediate the guidance of migrating cells. In this study, we show that Hoxb8a is a critical component in a network required for directional migration of the lateral line primordium, in which it acts downstream of Wnt signaling to control the spatial expression of the chemokine receptors, *cxcr4b* and *cxcr7b*.

Results

Hox Genes Are Expressed in the Leading Zone of the Migrating Primordium. Searches of the ZFIN database (http://zfin.org) identified several *hox* genes expressed in the posterior lateral line primordium: *hoxb8a, hoxc8a, hoxb6a, hoxc6a, hoxc4a*, and *hoxd4a*. We therefore analyzed their expression pattern in detail by whole mount in situ hybridization (ISH) during the formation and migration of the primordium, from 18 to 48 h postfertilization (hpf). Only *hoxb8a* and *hoxb6a* mRNAs were detected in the primordium before the onset of migration, which occurs at 22 hpf (28). At early stages, both genes were expressed in the leading two-thirds of the primordium, with an anterior limit of expression located in

L1, the first forming proneuromast (2) (Fig. 1*A* and Fig. S1). During migration, from 24 to 48 hpf, *hoxb8a* expression was maintained in the leading zone and down-regulated in the trailing zone and deposited cells (Fig. 1 *B* and *C* and Fig. S1). The *hoxb6a* showed a similar expression pattern initially but was not expressed after 35 hpf (Fig. S1). The other *hox* genes were up-regulated at later stages during migration, with similar expression in the leading zone of the primordium (Fig. S1). These expression patterns prompted us to examine potential roles of *hox* genes in the migrating primordium.

Hoxb8a Knockdown Leads to Posterior Lateral Line Defects. As several *hox* genes are coexpressed in the migrating primordium, they potentially have redundant functions. To investigate their role, we carried out antisense morpholino (Mo) knockdowns of *hoxb8a* and *hoxb6a,* the only *hox* genes expressed before the onset of migration. Splice-blocking morpholinos were designed

and their efficiency ascertained by RT-PCR (Fig. S2). We found similar effects of single knockdown of these genes on primordium migration (Fig. S2), but double knockdown had major effects on embryo morphology, likely reflecting roles in axial patterning. We therefore focused our analysis on *hoxb8a* as it is expressed throughout the migration process (Fig. 1 *A*−*C* and Fig. S1). Following knockdown of *hoxb8a,* decreased migration of the primordium was observed at 45 hpf (Fig. 1 *D* and *E*). Time-lapse imaging from 24 hpf (Movie S1) and quantification of the distance traveled by the primordium at 26 hpf (Fig. S2) revealed a decreased speed during the early phase of migration. Later on, the delayed primordia either continued to migrate at reduced speed to the tip of the tail or stalled in the second half of the trunk region. These changes to migration of the primordium were not due to altered *sdf1a* expression, as this was unaffected by *hoxb8a* knockdown (Fig. S3). Similar lateral line phenotypes were observed in embryos injected with an ATG-blocking *hoxb8a* Mo, and as shown below, the specificity is corroborated by other loss of function approaches. In addition to changes in migration, we observed a decreased cell number in the primordium of Hoxb8a morphant embryos, and reduction of BrdU incorporation at 24 hpf (Fig. S4). Taken together, these results show that Hoxb8a is required for the normal speed and distance of migration, as well as for normal proliferation of primordium cells.

Hoxb8a Is Required for *cxcr4b* Expression in the Primordium. To understand why migration is affected in *hoxb8a* morphants, we analyzed the expression of *cxcr4b* and *cxcr7b* that are required for directional migration. We found that expression of *cxcr7b* was unchanged in *hoxb8a* morphants (Fig. 1*G*), but a marked reduction in *cxcr4b* expression was detected at 30 hpf (60% of morphants, $n = 84$) compared with control Mo injected embryos (Fig. 1*F* and Fig. S2). A similar decrease in *cxcr4b* expression was observed in embryos injected with the ATG-blocking *hoxb8a* Mo. We conclude that Hoxb8a is required for normal expression of *cxcr4b* in the primordium. In view of the crucial role of *cxcr4b* in directional migration, the decrease in its expression could account for the migration delay in *hoxb8a* morphants.

One potential explanation of these findings is that Hoxb8a knockdown perturbs the Wnt/Fgf network that is required for correct patterning and migration of the primordium. To investigate this, we analyzed the expression of *lef1* and *pea3*, which are readouts of Wnt and Fgf signaling, respectively (13, 16, 17). The expression of both genes appeared unchanged in *hoxb8a* morphants (Fig. 1*H* and Fig. S5). Moreover, injection of *hoxb8a* Mo did not affect the expression of *fgf10*, *fgf3*, and the Fgf inhibitor *sef1* that are regulated by Wnt signaling (13), or of *fgfr1*, a target of the Fgf pathway (13) (Fig. S5). These data indicate that the decreased *cxcr4b* expression and primordium migration upon Hoxb8a knockdown are not due to an alteration in Wnt and Fgf signaling pathways. Consistent with this, the formation and morphology of neuromasts were normal in *hoxb8a* morphants (Fig. 1*D*, Fig. S2, and Movie S1).

Overexpression of Hoxb8a Throughout the Primordium Down-Regulates *cxcr7b*. These findings raised the question of why *hox* gene expression is restricted to the leading region of the primordium and down-regulated in the most mature proneuromast before deposition. To address this, we overexpressed Hoxb8a throughout the primordium and deposited cells. A Myc-tagged full-length Hoxb8a protein (Hoxb8aMT) was used to generate a *UAS:Hoxb8aMT* line, which was crossed with a *cldnb4.2kb:gal4* line in which Gal4 (29) is expressed under control of the *cldnb* proximal promoter. This Gal4 line drives continuous and mosaic expression of UAS-driven genes in the primordium and deposited cells from 18 to 20 hpf and throughout the migration (Fig. S6). A potential complicating factor is that transgene expression from the *cldnb* promoter also occurs in surface ectoderm (5), so we analyzed the possibility of a nonautonomous effect on *sdf1a* expression in the somites; however, we found no such change after expression of normal or mutant Hoxb8a.
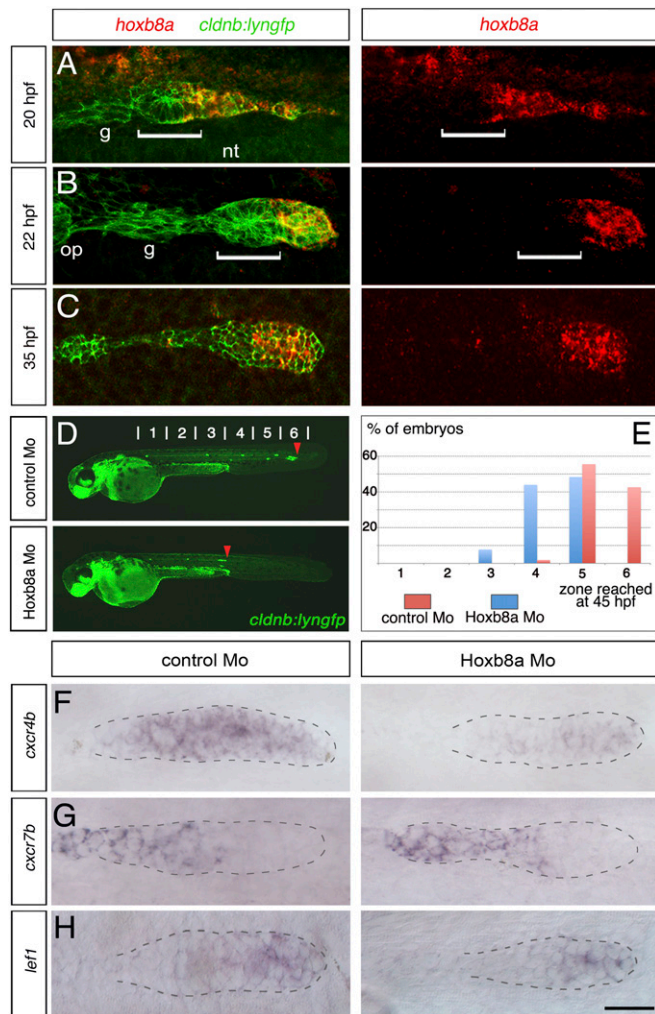

Fig. 1. *hoxb8a* is required for proper migration of the posterior lateral line primordium and expression of the chemokine receptor *cxcr4b*. Anterior to the left. Detection of *hoxb8a* mRNA (red) in *cldnb:lyngfp* embryos (green) at (*A*) 20, (*B*) 22, and (*C*) 35 hpf. Dorsal view in *A*, lateral views in *B* and *C*. White brackets indicate the width of the first forming proneuromast (L1). (*D*) Fuorescence images of *cldnb:lyngfp* embryos at 45 hpf injected with control and *hoxb8a* Mo. Red arrowheads indicate the position of the primordium. (*E*) Quantification of the migration of the primordium at 45 hpf (control Mo, $n = 54$; *hoxb8a* Mo, $n = 91$). ISH to detect expression of (*F*) *cxcr4b*, (*G*) *cxcr7b*, and (*H*) *lef1* at 30 hpf. The primordium is outlined with dotted line. g, Posterior lateral line sensory ganglion; op, otic placode; nt, neural tube. (Scale bar: 25 μm.)

Embryos overexpressing Hoxb8aMT in the posterior lateral line showed a major delay in migration, with the primordium stalling in the middle of the trunk at around 30 hpf (Fig. 2 *A* and *B*). Live imaging revealed that these primordia have uncoordinated collective migration (Movie S2), a behavior previously observed when *cxcr7b* function is impaired (7, 13). To further investigate the cause of this migration defect, we analyzed *cxcr4b* and *cxcr7b* expression. Hoxb8aMT overexpression did not affect *cxcr4b* expression in the primordium nor activate its expression in the deposited cells, but was sufficient to down-regulate *cxcr7b* in the back of the primordium and deposited cells (Fig. 2 *C* and *D* and Fig. S7). A loss of *cxcr7b* expression was previously observed upon expansion of Wnt activity throughout the primordium (13). However, *lef1* expression was still restricted to the leading zone, as in the control situation (Fig. 2*E*), indicating that *cxcr7b* down-regulation is not due to expanded Wnt activity. We conclude that Hoxb8a in the leading zone restricts *cxcr7b* expression to the trailing part of the primordium, and that down-regulation of *hoxb8a* in the trailing zone is therefore necessary for normal migration. However, Hoxb8a overexpression is not sufficient to up-regulate *cxcr4b* in the trailing zone.

A Dominant Repressor Form of Hoxb8a Down-Regulates *cxcr4b* and *cxcr7b* Expression. Hox proteins can act as transcriptional activators or repressors, depending on the context and nature of available cofactors (22, 23, 30). To further investigate Hoxb8a function, we generated a stable transgenic line expressing a dominant repressor form of Hoxb8a (fusion with the Engrailed repressor domain) (31) under the control of UAS sequences (*UAS:Hoxb8aEnRHA*). Overexpression of this repressor form in the primordium by use of the *cldnb4.2kb:gal4* line was found to result in strong migration defects (Fig. 3 *A* and *B*). In live imaging experiments, the primordia showed major defects in their migration, with stretched morphology, reminiscent of phenotypes observed in *cxcr4b* mutant and *cxcr7b* morphant embryos (Movies S3 and S4) (5, 7). Consistent with these observations, the
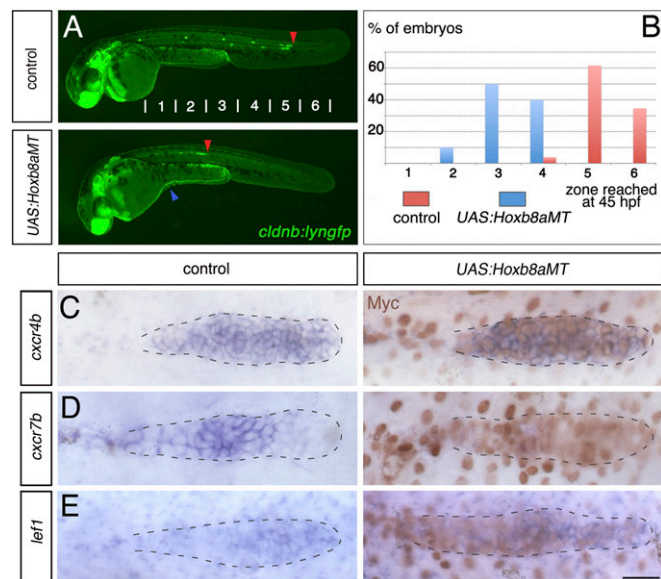

Fig. 2. Overexpression of Hoxb8a leads to down-regulation of *cxcr7b*. (*A*) Fluorescent images of *cldnb:lyngfp* in *cldnb4.2:gal4* x *UAS:Hoxb8aMT* embryos and control siblings at 45 hpf. The blue arrowhead shows the yolk defect seen in all *cldnb4.2:gal4* x *UAS:Hoxb8aMT* embryos. Red arrowheads indicate the position of the primordium. (*B*) Quantification of the migration at 45 hpf (control, n = 20; *cldnb4.2:gal4* x *UAS:Hoxb8aMT*, n = 26). Expression of (*C*) *cxcr4b*, (*D*) *cxcr7b*, and (*E*) *lef1* in *cldnb4.2:gal4* x *UAS:Hoxb8aMT* and control siblings at 28 hpf. ISH was followed by Myc immunostaining (brown). (Scale bar: 25 µm.)
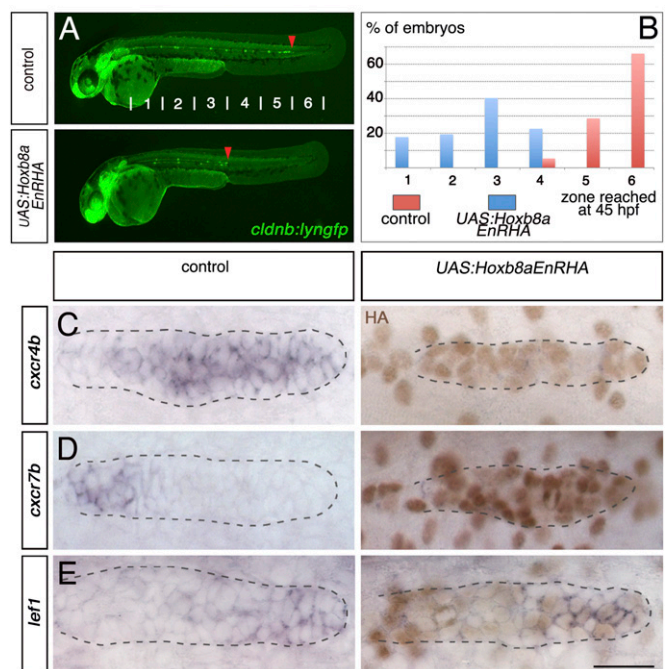

Fig. 3. Dominant repressor Hoxb8a down-regulates the expression of *cxcr4b* and *cxcr7b*. (*A*) Fluorescent images of *cldnb:lyngfp* in *cldnb4.2:gal4* x *UAS:Hoxb8aEnRHA* embryos and control siblings at 45 hpf. Red arrowheads indicate the position of the primordium. (*B*) Quantification of the migration at 45 hpf (control, n = 56; *cldnb4.2:gal4* x *UAS:Hoxb8aEnRHA*, n = 62). Expression of (*C*) *cxcr4b*, (*D*) *cxcr7b*, and (*E*) *lef1* in *cldnb4.2:gal4* x *UAS:Hoxb8aEnRHA* and control siblings at 30 hpf. ISH was followed by HA immunostaining (brown). (Scale bar: 25 µm.)

expression of both chemokine receptors was down-regulated by the dominant repressor form of Hoxb8a (Fig. 3 *C* and *D* and Fig. S8). In contrast, *lef1* expression appeared to be normal (Fig. 3*E*), and thus the decreased expression of the chemokine receptors is not due to perturbation of Wnt signaling. These results further implicate Hoxb8a in regulation of *cxcr4b* and *cxcr7b*.

We noticed a reduction in cell number within the primordium upon overexpression of the dominant repressor form of Hoxb8a (Fig. S9). By analyzing embryos in which transgene expression is mosaic, we found that Hoxb8aEnRHA cell-autonomously reduces BrdU incorporation in the primordium at 24 hpf (Fig. S9), further supporting that Hoxb8a is also involved in the control of cell proliferation.

A Dominant Activator Form of Hoxb8a Activates *cxcr4b* Expression and Inhibits *cxcr7b* Expression. To further investigate how Hoxb8a functions, we generated a stable transgenic line expressing a dominant activator form (fusion with the VP16 activator domain) (31) under the control of UAS sequences (*UAS:Hoxb8aVP16*). Targeted overexpression of Hoxb8aVP16 mediated by the *cldnb4.2:gal4* line resulted in a delay in primordium migration (Fig. 4 *A* and *B* and Movie S5). Strikingly, Hoxb8aVP16 overexpression led to an expansion of *cxcr4b* expression into the trailing zone and deposited cells at 28 hpf (Fig. 4*C*). Because full-length Hoxb8a was not sufficient to up-regulate ectopic *cxcr4b* expression (Fig. 2*C* and Fig. S7), the dominant activator form may override a need for a cofactor that is absent in the trailing zone. In contrast, the level of *cxcr7b* transcripts was reduced, although not completely abolished (Fig. 4*D*). The altered expression of chemokine receptors is likely to account for the delayed migration of the primordium (see *Discussion*). *Lef1* expression was normal (Fig. 4*E*), confirming the uncoupling between *cxcr4b*/*cxcr7b* regulation by Hoxb8a and overall Wnt activity.
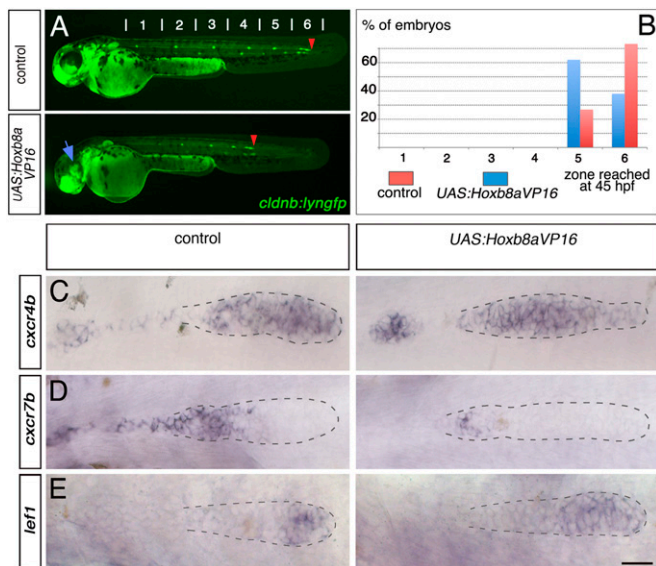
Fig. 4. Dominant activator Hoxb8a activates *cxcr4b* expression and down-regulates *cxcr7b*. (*A*) Fluorescent images of *cldnb:lyngfp* in *cldnb4.2:gal4* x *UAS:Hoxb8aVP16* embryos and control siblings at 45 hpf. Blue arrow shows the morphological eye defect observed in all Hoxb8aVP16 embryos. Red arrowheads indicate the position of the primordium. (*B*) Quantification of the migration at 45 hpf (control, n = 50; *cldnb4.2:gal4* x *UAS:Hoxb8aVP16*, n = 56). ISH showing expression of (*C*) *cxcr4b*, (*D*) *cxcr7b*, and (*E*) *lef1* in *cldnb4.2:gal4* x *UAS:Hoxb8aVP16* and control siblings at 28 hpf. (Scale bar: 25 μm.)

Hoxb8a Down-Regulates *cxcr7b* Expression Downstream of Wnt Signaling. Because ectopic activation of the Wnt pathway results in expansion of *cxcr4b* expression and loss of *cxcr7b* in the trailing primordium (13, 16), localized Wnt activity is thought to underlie the polarized expression of *cxcr4b* and *cxcr7b*. Our findings suggest that Hoxb8a does not act upstream of Wnt pathway activation, as *lef1* expression appears unaffected following loss or gain of Hoxb8a function. To investigate whether Hoxb8a is a target of the Wnt pathway, we inhibited Wnt signaling using the *hsp70:dkk1gfp* line (32), as verified by the down-regulation of *lef1* (Fig. 5 *A* and *B*). Overexpression of Dkk1 from 24 hpf led to a complete down-regulation of *hoxb8a* and *hoxb6a* expression at 30 hpf (Fig. 5 *C–F*), indicating that both *hox* genes are transcriptional targets of Wnt signaling in the leading part of the primordium.

In previous studies, inactivation of the Wnt pathway with the *hsp70:dkk1gfp* line led to an expansion of *cxcr7b* expression into the leading zone and no apparent change in levels of *cxcr4b* expression (10, 13), suggesting that *cxcr4b* expression is not regulated by the Wnt pathway. We found that 3 h after heat shock induction of dkk1, there was a major down-regulation in *lef1*, *hoxb6a*, and *hoxb8a* expression, with a mild decrease in *cxcr4b* and spreading of *cxcr7b* expression (Fig. S10), which became robust by 6 h (Fig. 5 *G* and *H*). Our results thus reveal that, in addition to confining *cxcr7b* expression to the trailing zone, Wnt activity is also required for long-term maintenance of normal *cxcr4b* expression levels in the primordium.

Our findings show that the Wnt pathway regulates Hoxb8a expression, which in turn regulates *cxcr4b* and *cxcr7b*. This raises the question of whether Hoxb8a is sufficient to rescue the effect of blocking Wnt activity on chemokine receptor expression. To investigate this, we crossed *hsp70:dkk1gfp/cldnb4.2:gal4* double transgenic fish with *UAS:Hoxb8aMT* or *UAS:Hoxb8aVP16* fish. In triple transgenic embryos, Wnt signaling is inhibited upon heat shock, and Hoxb8aMT or Hoxb8aVP16 are present in the primordium due to forced expression. Embryos were heat-shocked at 24 hpf, and Dkk1gfp positive and negative embryos analyzed at 30 hpf. We found that overexpression of Hoxb8aVP16, but not

Hoxb8aMT, restores normal levels of *cxcr4b* expression when Wnt signaling is inhibited (compare Fig. 5 *K* and *L* to *H*). This suggests that Hoxb8a needs a Wnt-dependent cofactor for *cxcr4b* activation, whose requirement is bypassed in the dominant activator form. Strikingly, in *hsp70:dkk1* embryos, *cxcr7b* expression was abolished by Hoxb8aMT (compare Fig. 5 *M* and *J*), and decreased by Hoxb8aVP16 (compare Fig. 5 *N* and *J*). Hoxb8a or its dominant activator form are therefore sufficient to down-regulate *cxcr7b* in the absence of Wnt signaling.

Discussion

Our findings have unraveled a regulatory network between Wnt signaling, a Hox transcription factor, and chemokine receptors, which is required for migration of the posterior lateral line primordium. Wnt signaling activates Hoxb8a expression in the leading zone of the primordium, which in turn maintains normal levels of *cxcr4b* expression and restricts *cxcr7b* expression to the trailing zone. Hoxb8a is thus a critical component in the network that provides directionality for collective cell migration.
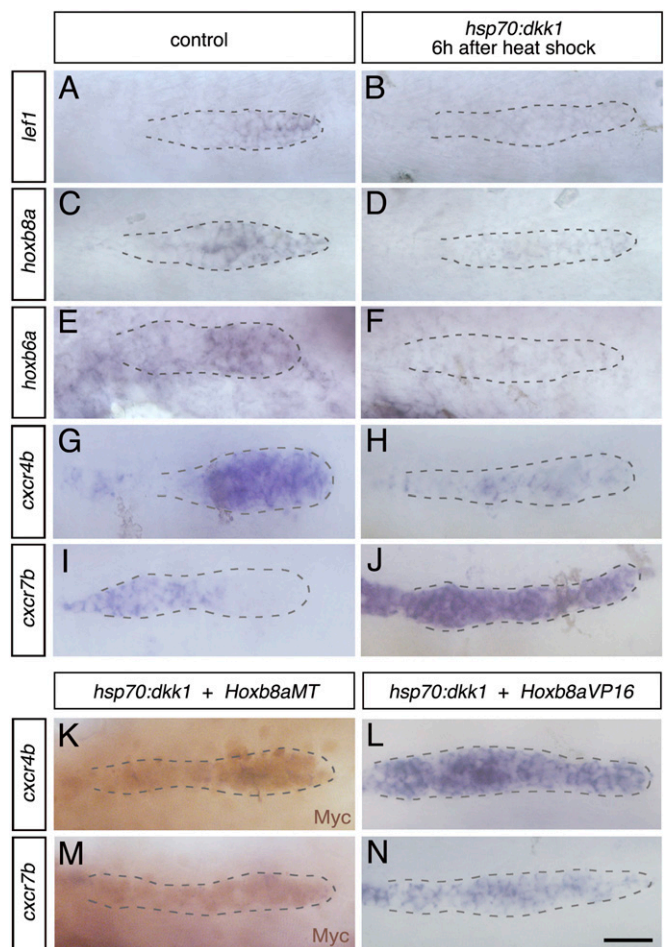

Fig. 5. Hoxb8a is a target of Wnt signaling and is sufficient to down-regulate *cxcr7b* upon inhibition of Wnt pathway. ISH showing expression of (*A* and *B*) *lef1*, (*C* and *D*) *hoxb8a*, (*E* and *F*) *hoxb6a*, (*G* and *H*) *cxcr4b*, and (*I* and *J*) *cxcr7b* in *hsp70:dkk1gfp* embryos and control heat-shocked siblings. Embryos were heat-shocked at 24 hpf and fixed at 30 hpf. ISH to detect expression of (*K*) *cxcr4b* and (*M*) *cxcr7b* followed by Myc immunostaining (brown) in *hsp70:dkk1gfp/cldnb4.2:gal4* x *UAS:Hoxb8aMT* embryos. ISH to detect expression of (*L*) *cxcr4b* and (*N*) *cxcr7b* in *hsp70:dkk1gfp/cldnb4.2: gal4* x *UAS:Hoxb8aVP16* embryos. The number of embryos indicated are pooled from three independent experiments and they are as follows: *A*, 57; *B*, 60; *C*, 62; *D*, 58; *E*, 41; *F*, 63; *G*, 66; *H*, 40; *I*, 67; *J*, 67; *K*, 23; *L*, 55; *M*, 38; *N*, 46. (Scale bar: 25 μm.)

DEVELOPMENTAL BIOLOGY

Hoxb8a Mediates Differential Expression of *cxcr4b* and *cxcr7b*. Based on finding that several *hox* genes are expressed in the developing posterior lateral line in a dynamic and restricted fashion, we investigated the function of *hoxb8a* with gain and loss of function approaches. Our findings, summarized in Fig. 6*A*, reveal that Hoxb8a is involved in the differential regulation of the chemokine receptors and in cell proliferation in the primordium. Hoxb8a is required to maintain normal levels of *cxcr4b* expression in the primordium, and this involves transcriptional activation, because dominant repressor and activator forms of Hoxb8a abolish and ectopically activate *cxcr4b* expression, respectively. The *cxcr4b* expression is not completely abolished in Hoxb8a morphants, and this could be due to incomplete knockdown of Hoxb8a (Fig. S2) and/or functional overlap with coexpressed *hox* genes or Lef1 (9). These factors may also contribute to functional overlap in regulation of *cxcr7b* expression, which does not expand toward the leading zone in *hoxb8a* morphants, whereas it is repressed by Hoxb8a overexpression.

The effects of Hoxb8a dominant activator and repressor overexpression suggest a complex relationship between Hoxb8a and *cxcr7b* expression. We find that Hoxb8a dominant repressor downregulates *cxcr7b* expression, which seems to be a specific effect, as *lef1* (Fig. 3*E*), *eya1* (Fig. S8), and *cldnb:lyngfp* transgene (Fig. 3*A*)

Fig. 6. Summary and working model for the regulation of *cxcr4b* and *cxcr7b* by Hoxb8a. (*A*) Summary of changes in the expression of *cxcr4b* and *cxcr7b* following experimental manipulations. (*B*) Proposed working model. In the leading primordium, Wnt signaling activates the expression of Hoxb8a, which in turn regulates the chemokine receptors *cxcr4b* and *cxcr7b*. Hoxb8a is likely to activate *cxcr4b* with a Wnt-dependent cofactor that could be Lef1. Hoxb8a down-regulates *cxcr7b* through two possible mechanisms: activation of a repressor X and repression. Hoxb8a also regulates cell proliferation within the primordium and another branch downstream of Wnt signaling controls proliferation via Lef1.

expression are not affected. This finding argues that *cxcr7b* downregulation involves Hoxb8a-mediated transcriptional repression. Intriguingly, overexpression of a dominant active form of Hoxb8a also led to down-regulation of *cxcr7b* expression. The simplest explanation is that Hoxb8a acts through two parallel mechanisms to restrict *cxcr7b* expression to the trailing zone, one involving Hoxb8a-mediated repression and the other the activation of an unknown repressor (Fig. 6*B*).

Inhibition of Fgf signaling and/or activation of the Wnt pathway lead to concurrent *cxcr7b* down-regulation and expansion of *cxcr4b* expression into the trailing zone, both of which may contribute to the strong migration defects seen in these situations (13, 16). It is informative to compare these results with the effects of manipulating Hoxb8a function. In Hoxb8aMT-overexpressing embryos, *cxcr7b* expression is abolished but *cxcr4b* expression is not expanded, and migration of the primordium is strongly affected. Migration defects are weaker in Hoxb8aVP16-overexpressing embryos, in which *cxcr7b* expression is only partly decreased and *cxcr4b* expression is expanded. Thus, the severity of the migration delay correlates with the decrease in *cxcr7b* expression levels rather than with expansion of *cxcr4b* expression. These findings are consistent with the major role of *cxcr7b* in directional migration, in which it is proposed to create anisotropy in Cxcr4b activation by acting as a sink for Sdf1a (8).

Hoxb8a Acts Downstream of Wnt Signaling. Because the polarized distribution of *cxcr4b* and *cxcr7b* expression is altered upon modulation of Wnt signaling, Hoxb8a may act downstream of Wnt activity to regulate their expression. Indeed, we found that *hoxb8a* (and *hoxb6a*) expression is abolished upon Wnt inhibition, showing that *hoxb8a* is a direct or indirect transcriptional target of the Wnt pathway in the leading zone (Fig. 6*B*). Whereas there was a major decrease in *lef1* and *hoxb8a* expression 3 h after Dkk1 induction, *cxcr4b* expression had only a mild decrease at this stage, but was strongly down-regulated by 6 h. This slower change in *cxcr4b* expression may reflect perdurance of Hoxb8a protein and/or of *cxcr4b* mRNA after Dkk1 induction. Although previous studies (10, 13) had not observed decreased *cxcr4b* expression after blocking Wnt activity, this is likely explained by these studies having analyzed expression at earlier stages after Dkk1 induction.

Hoxb8a overexpression is sufficient to down-regulate *cxcr7b* in the presence or absence of Wnt signaling. In contrast, in the context of Wnt pathway inhibition, dominant activator but not full-length Hoxb8a is able to rescue normal *cxcr4b* levels. Together with the observation that dominant activator but not full-length Hoxb8a is sufficient to activate *cxcr4b* in the trailing zone and deposited cells, these findings suggest a model in which Wnt signaling is required for leading zone expression of both Hoxb8a and a cofactor required for up-regulation of *cxcr4b* (Fig. 6*B*). This cofactor could be Lef1, as this component of the Wnt pathway contributes to activation of a minimal *cxcr4b* promoter (9). Because *lef1* knockdown does not affect *cxcr4b* expression, it acts in parallel with other factors that compensate for its function (9). Taken together, this suggests a feed-forward mechanism in which Wnt signaling acts by inducing and cooperating with Hoxb8a in the regulation of *cxcr4b* (Fig. 6*B*). These findings set the stage for investigating the relationship between Hoxb8a and other factors that regulate *cxcr4b* and *cxcr7b* expression (9, 33).

Experimental Procedures

Fish Strains and Transgenesis. Embryos were obtained by natural spawning and raised at 28.5 °C (34). The primordium was visualized using the *cldnb:lyngfp* line (5), and the *hsp70:dkk1gfp* line (32) used to inhibit Wnt signaling. The *cldnb4.2:gal4* line (nim11) was generated by placing the Gal4 sequence (29) downstream of 4.2 kb of the proximal promoter of the *cldnb* gene. For overexpression, full-length Hoxb8a was tagged with 6xMyc at the C terminus (Hoxb8aMT). Hoxb8a dominant repressor and activator forms were generated by fusing Engrailed repressor (HA tagged, Hoxb8aEnRHA) or VP16 activator (Hoxb8aVP16) domains (31), respectively, C-terminal to full-length Hoxb8a sequence. Hoxb8aMT, Hoxb8aEnRHA, and Hoxb8aVP16 were placed under the control of UAS regulatory sequences and used to establish
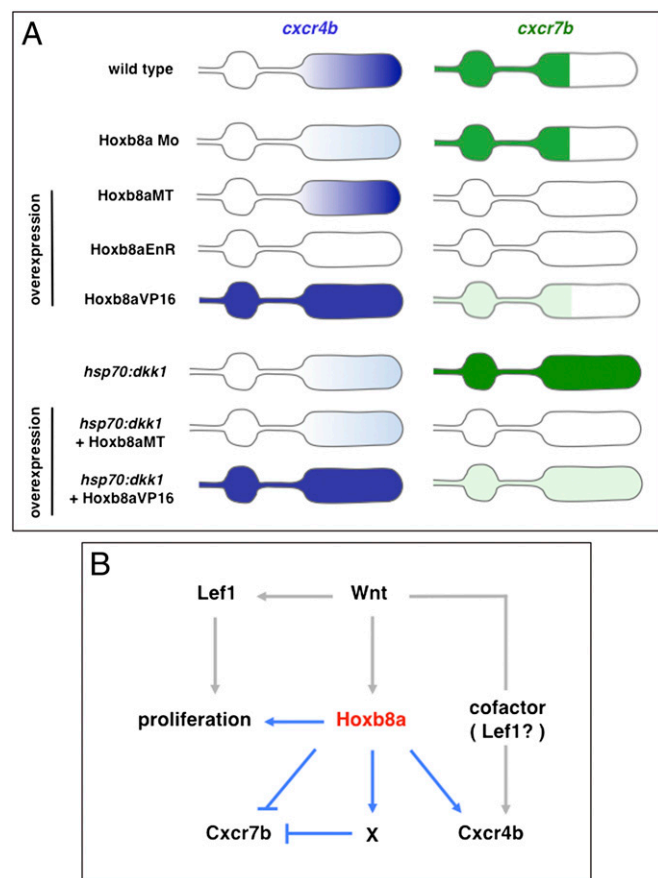
stable transgenic lines (nim12, -13, and -14, respectively) using Tol2 mediated integration (35).

In Situ Hybridization and Immunostaining. Whole mount ISH with digoxigenin-labeled RNA probes was performed as described (36). Detection with fluorescent substrate used the Tyramide TSA-Plus Palette System (Perkin-Elmer). The size of the chemokine receptor expression domains has been shown to change during the deposition cycle of the primordium (12). For *cxcr4b* and *cxcr7b* ISH, care was taken to compare primordia with similar deposition states. Immunostaining used the following primary antibodies: anti-GFP (rabbit, Torrey Pines Biolabs), anti-Myc (mouse, clone 9E10, Santa Cruz), anti-HA (rat, Roche), anti-VP16 (mouse, Santa Cruz), and anti-BrdU (mouse, Sigma). For some experiments, Myc or HA immunostainings were performed after ISH; this did not work for VP16 immunostaining. To identify Hoxb8aMT-, Hoxb8aEnRHA-, or Hoxb8aVP16-expressing embryos among their siblings, we used the yolk morphology defect (Fig. 2A), the overall reduction in embryo size and eye morphology defect (Figs. 3A and 4A), or ectopic expression of *cxcr4b* in the olfactory placode (Fig. S11), respectively.

Morpholino Knockdown and Heat-Shock Inductions. Antisense Mo were obtained from Gene Tools LLC. Four to ten nanograms of Mo were coinjected with 8 ng p53 Mo (37, 38). With doses exceeding 7 ng, injections of *hoxb8a* Mo led to some embryos with morphological alterations combined with somitic defects and disruption of Sdf1a expression, likely due to early functions of Hoxb8a. We therefore used a dose of 5 ng, leading to a partial knockdown of the gene (Fig. S2), such that embryo morphology (Fig. 1D) was not affected. Morpholino sequences used are as follows:

Control Mo: 5′ CCTCTTACCTCAGTTACAATTTATA 3′

Hoxb8a ATG Mo: 5′AGCTCATCTTTTACTGCTGTTGGTG 3′

Hoxb8a splice blocker (SB) Mo: 5′ TTTCTGTCTCACCTTGAGGTCGCAT 3′

Hoxb6a SB Mo: 5′ TTACCGAAGGTCCCTGTCCATGAGA 3′

p53 Mo: 5′ GCGCCATTGCTTTGCAAGAATTG 3′

To inhibit Wnt signaling, *hsp70:dkk1gfp* (32) embryos were heat-shocked at 24 hpf for 30 min at 37 °C and fixed at 30 hpf for analysis.

Confocal and Time Lapse Imaging. *Cldnb:lyngfp* embryos were dechorionated manually, immobilized in 0.005% Tricaine (Sigma), and mounted in 3% (wt/vol) Methylcellulose in 0.5X Danieau's solution. Movies were recorded at 24–25 °C on a Leica DMIRE2 SP2 upright confocal microscope.

BrdU Incorporation. Embryos were incubated at 28.5 °C in 10 mM BrdU or 10% (vol/vol) DMSO at 24 hfp for 1 h, washed and fixed, and then processed for BrdU immunostaining.

Statistical Analysis. Graphs show means ± sem (SD of the mean). *P* values correspond to two-tailed Student *t* test analysis. For quantification of the migration at 45 hpf, the trunk of the embryo was subdivided into six zones of equal size (Fig. 1D). For each zone, the proportion of embryos in which the primordium had reached the zone was calculated and plotted.

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