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# Evolution of Genetic Networks Underlying the Emergence of Thymopoiesis in Vertebrates

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## SUMMARY

About 500 million years ago, a new type of adaptive immune defense emerged in basal jawed vertebrates, accompanied by morphological innovations, including the thymus. Did these evolutionary novelties arise de novo or from elaboration of ancient genetic networks? We reconstructed the genetic changes underlying thymopoiesis by comparative genome and expression analyses in chordates and basal vertebrates. The derived models of genetic networks were experimentally verified in bony fishes. Ancestral networks defining circumscribed regions of the pharyngeal epithelium of jawless vertebrates expanded in cartilaginous fishes to incorporate novel genes, notably those encoding chemokines. Correspondingly, novel networks evolved in lymphocytes of jawed vertebrates to control the expression of additional chemokine receptors. These complementary changes enabled unprecedented Delta/Notch signaling between pharyngeal epithelium and lymphoid cells that was exploited for specification to the T cell lineage. Our results provide a framework elucidating the evolution of key features of the adaptive immune system in jawed vertebrates.

## INTRODUCTION

Adaptive immune systems provide anticipatory, clonally distributed, diverse, and self-tolerant repertoires of antigen receptors for immune defense. To date, adaptive immune systems have only been described in vertebrates (Cooper and Alder, 2006). Interestingly, adaptive immune systems appear to have independently arisen twice in basal vertebrates, once in the lineage that gave rise to jawless vertebrates, with hagfish and lamprey as their surviving representatives, and a second time in the lineage giving rise to all jawed vertebrates, with cartilaginous fishes (sharks, rays, skates, and so-called chimaeras) as their most ancestral extant forms. Jawless vertebrates utilize gene conversion (Nagawa et al., 2007) to generate diversity of their variable lymphocyte receptors (Pancer et al., 2004), while jawed vertebrates employ the so-called VDJ recombination (Schatz, 2004) in developing lymphocytes to achieve combinatorial diversity of immunoglobulins and T cell receptors respectively (Litman and Cooper, 2007). The fundamental differences in the mechanisms of recombination and the molecular nature of receptor structures highlight the independent origins of these two types of adaptive immune systems. Yet, both types of rearranging systems function in lymphocytes, suggesting that the common ancestor of vertebrates possessed lymphocytes but no adaptive immune system. The adaptive immune system of jawed vertebrates emerged in cartilaginous fishes about 500 million years ago. In addition to the primordial myelolymphoid lineage of lymphocytes (Kawamoto, 2006), the adaptive immune system of jawed vertebrates is distinguished by the emergence of T cells as a novel lymphocyte lineage, and the thymus as a primary lymphoid organ (Boehm and Bleul, 2007). Functionally, T cells and thymus are inextricably linked, for without the latter, T cell development fails.

Thymopoiesis is a complex process involving colonization of the epithelial organ anlage by lymphoid progenitor cells (Bhandoola et al., 2007), commitment of these progenitors to the T cell lineage (Hozumi et al., 2008; Koch et al., 2008), and their subsequent differentiation, including tolerance induction (Anderson et al., 2007). Differentiation of the prospective thymic epithelium in the pharyngeal endoderm (Rodewald, 2008) critically depends on the function of the transcription factor Foxn1 (Nehls et al., 1996). Mutations in the *Foxn1* gene are associated with a failure of thymopoiesis owing to a nonfunctional epithelial microenvironment (Blackburn et al., 1996; Nehls et al., 1996), which lacks expression of chemokine genes (Bleul and Boehm, 2000;

Itoi et al., 2007) that are thought to be important for attraction of lymphoid progenitors (Bleul and Boehm, 2000) and delta-like genes (Itoi et al., 2007) required for their specification toward the T cell lineage (Hozumi et al., 2008; Koch et al., 2008). Furthermore, *Foxn1* is required for the differentiation of epithelial progenitor cells (Bleul et al., 2006) into all subsets of the thymic epithelium, including those defining the cortex and the medulla (Bleul et al., 2006; Rossi et al., 2006). These observations indicate that *Foxn1* occupies a key position in the genetic network(s) establishing a functional thymic niche, which is essential for T cell development. Many genes are important for the early development of T cells, including, among others, *Notch1*, *Rbpj*, *Gata3*, *Ikaros*, and *Bcl11b* (for review, see Rothenberg et al., 2008), although their individual contributions to the sequential steps of T cell development are not always well documented. B cell development appears to be the default pathway of differentiation for lymphoid progenitor cells, unless Notch signaling (through Notch1 receptors; Radtke et al. [2004]) occurs in the thymic epithelial anlage, which expresses Dll4 as the essential and non-redundant ligand for T cell specification (Hozumi et al., 2008; Koch et al., 2008). These observations support earlier suggestions that antibody-producing myelolymphoid cells might represent the primordial phenotype of the vertebrate lymphocyte lineage (Kawamoto, 2006) and that the T cell lineage emerged at a later point (Boehm and Bleul, 2007). Unfortunately, neither jawless nor cartilaginous fishes, which occupy key phylogenetic positions with respect to the evolution of adaptive immune systems, are amenable to genetic analysis. Therefore, the elucidation of the genetic changes underlying the emergence of thymopoiesis poses a formidable challenge; yet, such studies might provide essential information for the understanding of cellular immunity. We set out to reconstruct the evolutionary origins of thymopoiesis, employing the following strategy. First, we analyzed the genomes of several key chordate and vertebrate species in order to trace the evolutionary history of genes potentially involved in the emergence of thymopoiesis. Second, we determined their possible coexpression in order to derive models of potential genetic interactions. Finally, we tested these predictions in bony fishes to validate the structure of relevant genetic networks. Our results indicate how in jawed vertebrates, ancient genetic networks in pharyngeal epithelium and lymphocytes increased in complexity and how these novel functionalities enabled unprecedented cellular interactions between these two cell types. We suggest that these key advances provided the basis for the evolution of T cells and the thymus.

## RESULTS

### Evolutionary History of *Foxn1*

*Foxn1* occupies a central position in the genetic network(s), establishing a functional thymic rudiment. The *Foxn1* gene is first identifiable in cartilaginous fishes and then found in all other jawed vertebrates (Figure 1). Detailed analyses of gene structures and derived protein sequences suggest that *Foxn1* is a paralog of *Foxn4* (Figure 1; Figures S1A and S1B available online). *Foxn4* itself first appears in cephalochordates (amphioxus), where it coexists with its evolutionarily more ancient paralog, *Foxn4b*, which appears to be absent from the genomes of urochordates and all vertebrates (Figures 1 and S1). Jawless fishes, which represent the most basal group of extant vertebrates, possess a gene very similar to *Foxn4*, designated *Foxn4-like* (*Foxn4L*). Analysis of derived protein sequences and short-range synteny relationships (Figure S1C) indicates that Foxn1 is the *Foxn4L* ortholog in jawed vertebrates (Figure 1). The genealogy of the *Foxn4*/*Foxn4L*/*Foxn1* lineage is supported by the expression patterns of these genes. In amphioxus, *Foxn4* (i.e., *Foxn4a*) is expressed in the pharyngeal endoderm (Figure S2), among other sites; in lamprey, *Foxn4L* is expressed in epithelia lining the gill basket (Figure 2A); and in cartilaginous fishes and all other jawed vertebrates, *Foxn1* is expressed in the thymus (Figure S2). The shared expression domains of *Foxn4* and its paralog *Foxn4L* in the pharynx suggest that expression of a *Foxn4L* ortholog also occurred in the pharyngeal epithelium of the common ancestor of vertebrates and thus antedates the evolutionary emergence of the thymus.

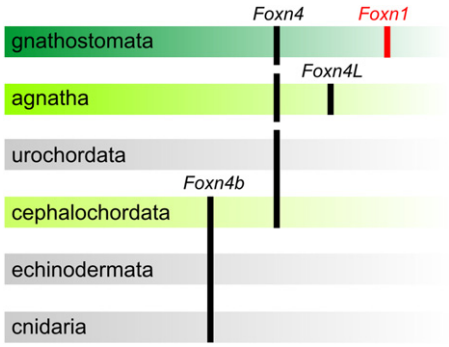



**Figure 1. Evolutionary History of *Foxn1*-like Genes**

*Foxn4b* represents an ancient eumetazoan gene that duplicated in cephalochordata to give rise to *Foxn4a*, which is orthologous to *Foxn4* in urochordates and all vertebrates. *Foxn4L* is a paralog of *Foxn4* arising in jawless fishes; *Foxn1* is a paralog of *Foxn4* arising in jawed vertebrates. For jawed vertebrates (gnathostomata), the following organisms were analyzed: mouse (*Mus musculus*), chicken (*Gallus gallus*), frog (*Xenopus tropicalis*), medaka (*Oryzias latipes*), catshark (*Scyliorhinus canicula*), and elephant shark (*Callorhinchus milii*). For jawless vertebrates (agnatha), the following organisms were analyzed: sea lamprey (*Petromyzon marinus*), European river lamprey (*Lampetra fluviatilis*), European brook lamprey (*Lampetra planeri*), and arctic lamprey (*Lampetra japonica*). For urochordata, the sea squirt (*Ciona intestinalis*) was analyzed. For cephalochordata, the common lancelet or amphioxus (*Branchiostoma lanceolatum*) and the Florida lancelet (*Branchiostoma floridae*) were analyzed. The purple sea urchin (*Strongylocentrotus purpuratus*) and the sea anemone (*Nematostella vectensis*) were analyzed as representatives of echinodermata and cnidaria, respectively. For further details, see Figure S1 and the Supplemental Data.

### *Foxn1* Functions Upstream of Delta-like Genes

The differentiation of lymphocyte progenitor cells into the T cell lineage depends on the Notch ligand Delta-like 4 (*Dll4*) (Hozumi et al., 2008; Koch et al., 2008). Given that the expression of *Dll4* in the mouse is absent in thymic epithelial cells lacking *Foxn1* (Itoi et al., 2007), we examined whether this functional relationship might have been established earlier in evolution. A phylogenetic analysis of *delta*-like genes in chordate genomes revealed that the amphioxus genome contains a single delta-like gene (Rasmussen et al., 2007), whereas the genomes of

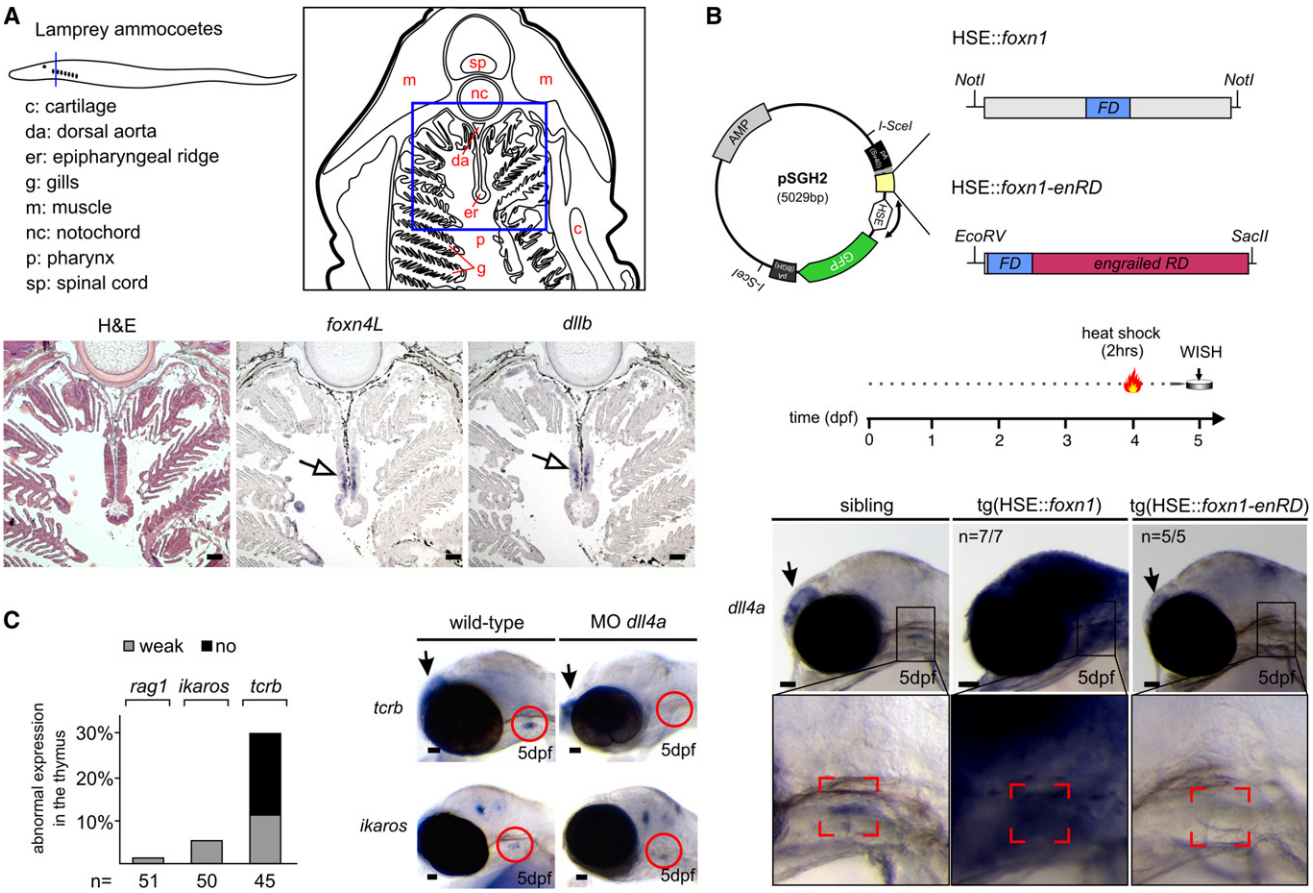



**Figure 2. *Foxn1*-like Genes Control Expression of *delta*-like Genes**

(A) Schematic indicating the plane of the section anterior of the second gill opening of *L. planeri* larvae (blue line) and the major anatomical landmarks in the section (upper panels); the blue rectangle corresponds to the sections shown in the lower panels. Coexpression (arrows) of *foxn4L* and *dllb* in the epipharyngeal ridge as detected by RNA in situ hybridization; a section stained with hematoxylin/eosin is shown for comparison (lower panels).

(B) Misexpression of wild-type *foxn1* (HSE::*foxn1*) or a dominant-negative version of *foxn1* (HSE::*foxn1-enRD*) from a heat-shock promoter regulates *dll4a* expression in transgenic medaka embryos. The upper panel indicates the structure of the bidirectional construct, wherein GFP expression can be used as a positive control for induction, the middle panel outlines the timing of the experiment, and the lower panel shows the expression patterns of *dll4a* and the number of embryos in which the indicated pattern was observed as an overview (upper panel) and detail (lower panel). The position of the thymus is highlighted in red; arrows point to the emx-1 expression domain in the brain.

(C) Interference with *dll4a* function does not affect homing to the thymic rudiment, as indicated by normal expression patterns for *ikaros* and *rag1*; by contrast, a substantial fraction of embryos lacks expression of *tcrb* expression as a sign of impaired T cell specification (left panel). Examples of embryos showing the specific effect of *dll4a* morpholinos (morpholino concentration 400 μM for all experiments) on *tcrb* and *ikaros* expression are shown in the right panel. The position of the thymus is highlighted in red; arrows point to the emx-1 expression domain in the brain.

Scale bars represent 50 μm.

jawless and cartilaginous fishes and mammals each contain three distinct genes; the genomes of bony fishes carry two extra copies (a total of five genes), presumably owing to their having undergone an additional round of genome duplication (Figure S3A; further details about this and all other phylogenetic analyses in this paper can be found in the Supplemental Data). Coexpression of *Foxn4a* and *Dll* genes occurs in the pharyngeal endoderm of the cephalochordate amphioxus (Figure S2). Moreover, one of the lamprey *delta-like* genes, *dllb* is expressed in a circumscribed region of the epithelium lining the gill basket of ammocoete larvae, precisely overlapping with the *Foxn4L* expression domain (Figure 2A). This strongly suggests an evolutionarily ancient pattern of coexpression of *Foxn4*/*Foxn4L* and *Delta-like* genes. To examine whether this coexpression is indicative of a functional interaction, we turned to bony fishes as the most basal genetically tractable vertebrate. Of the five *delta-like* genes in medaka fish (*O. latipes*), only *dll4a* was expressed in the thymic rudiment (Figure S3B). In order to determine directly whether *foxn1* is genetically upstream of *dll4a*, we derived two transgenic lines in medaka. The first line contains a full-length mouse *Foxn1* complementary DNA (cDNA) under the control of a heat-shock promoter; the second line contains a dominant-negative version of *Foxn1*, designated *foxn1*-enRD (Figure 2B). Induction of wild-type *foxn1* led to expression of *dll4a* in the embryo, indicating that *foxn1* augments *dll4a* expression; conversely, when *foxn1*-enRD was induced, *dll4a* expression in

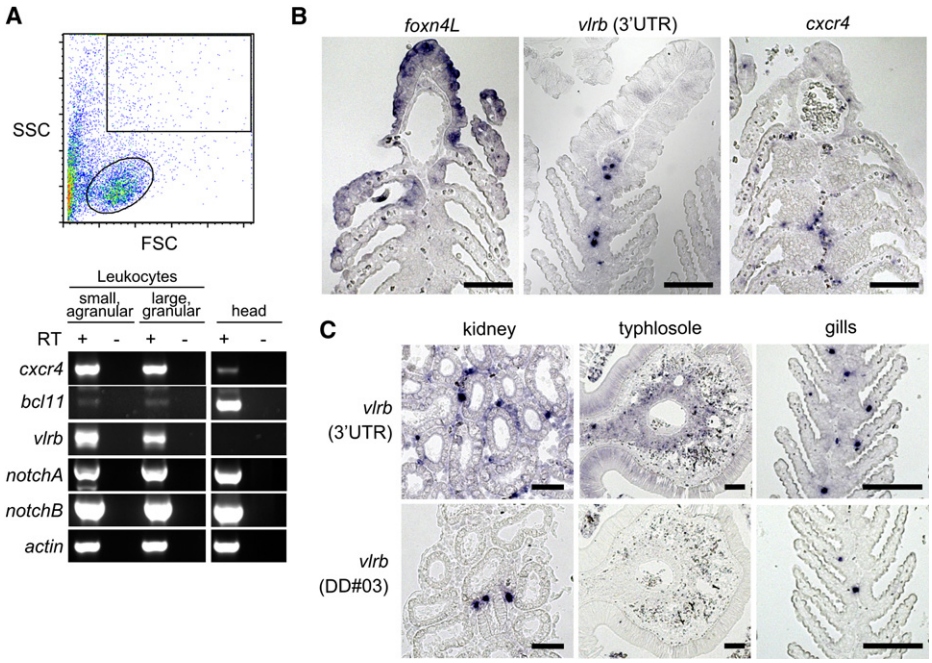



**Figure 3. Characterization of *L. planeri* Lymphocytes**

(A) Gene expression analysis (see Table S1 for details) by RT-PCR of leukocyte fractions purified by flow cytometry from kidney (small agranular [oval gate] cells represent lymphocytes; large granular [rectangular gate] cells represent lymphocytes and leukocytes). Results for reactions with (+) or without (−) reverse transcriptase (RT) are shown. RNA isolated from head tissues serves as a control.

(B) Leukocytes expressing *vlr* and *cxcr4* genes are located in the vasculature but not in the epithelium. Note that the expression level of bcl11a and *notch* genes in leukocytes is too low to be reliably detected by mRNA in situ hybridization.

(C) Lack of large clusters of lymphocytes in ammocoete tissues as assessed by antisense RNA probes detecting all (3′ UTR) or a subset (DD#03) of *vlr* genes. Scale bars represent 100 μm.

the thymic rudiment was abolished (Figure 2B). Hence, *foxn1* acts upstream of the *dll4a* gene in medaka. In order to examine whether *dll4a* is also required for T cell specification in this species, we first established that one of the Notch genes (Figure S4A), *notch 1b*, is expressed in the medaka thymus; however, knockdown of *notch1b* had pleiotropic effects (data not shown), precluding a meaningful interpretation of the resulting phenotype. By contrast, interference with expression of *dll4a* caused no general developmental defects, and a significant fraction of *dll4a* morphants showed no expression of the T cell receptor beta chain (*tcrb*) in thymocytes, whereas *ikaros* expression (as a marker of hematopoietic cells) was unaffected (Figure 2C). This indicates that T cell specification via delta-like ligands is an evolutionarily ancient function.

## No Evidence for a Thymus in Lamprey

Having established *foxn4L* and *dllb* as molecular tools to identify the location of a potential thymic microenvironment in lamprey, we examined whether this region of the gill basket also contained aggregates of leukocytes, as a morphological sign of a primordial lymphoid organ. To identify leukocytes in histological sections of ammocoete larvae of *Lampetra planeri*, we examined the expression patterns of two genes: variable lymphocyte receptor (*vlr*) genes (Pancer et al., 2004) are expressed in lymphocytes, and the *cxcr4* chemokine receptor (Kuroda et al. [2003]; for a phylogenetic analysis of chemokine receptors, see Figure S5) is expressed on virtually all leukocytes (Figure 3A). Cells expressing either of these two signature genes were not found within epithelial structures. In the gills, they are strictly intravascular (Figure 3B); moreover, no large aggregates of *vlr*- or *cxcr4*-positive cells could be detected in the kidney or in the typhlosole (Alder et al., 2008), the two major hematopoietic sites in ammocoete larvae (Figure 3C). This suggests that lamprey does not possess a lymphoid organ resembling the thymus of jawed vertebrates. Nonetheless, it was of interest to determine whether lymphocytes and other leukocytes express notch genes, which transmit delta-like signals to developing hematopoietic cells (Radtke et al., 2004). Indeed, the two notch genes identified in the lamprey genome (Figure S4A) are expressed in these cells (Figure 3A). Hence, our data suggest that although Notch receptor and ligand pairs are already expressed in the required cell types, lamprey lacks a facility required for bringing these two cell types into close proximity.

## Chemokine and Chemokine Receptor Genes in Basal Vertebrates

A distinguishing feature of T cells is their ability to home to epithelial structures (thymus anlage, mucosal linings, skin). Given the lack of lamprey lymphocytes at these sites, we hypothesized that lymphocytes in jawed vertebrates might have acquired additional facilities to accomplish this, conceivably via specific chemokines and chemokine receptors. To gain insight into this

process, we analyzed the genomes of amphioxus, a jawless fish, and a cartilaginous fish for evidence of such genes. The amphioxus genome lacks chemokine receptors (Nordström et al., 2008); the lamprey genome contains at least five chemokine receptor genes, including the known *cxcr4* gene (Figure S5A). Lamprey possesses two CC chemokine genes and a *cxcl12* homolog (Figure S6). An ortholog of *ccr9*, which, in the mouse, is implicated in facilitating the first wave of embryonic thymus colonization (Benz and Bleul, 2005; Liu et al., 2006), first appears in cartilaginous fishes (Figure S5A), as does the gene encoding its ligand, ccl25 (Figure S6). This suggests that bona fide chemokine and chemokine receptor genes first emerged in the genome of the common ancestor of vertebrates and that this probably coincided with the emergence of lymphocytes as a distinct cell lineage. These gene families then expanded in cartilaginous fishes and further increased in diversity in bony fish and higher vertebrates, coincident with the emergence of primary and secondary lymphoid tissues, additional hematopoietic cell lineages, and more complex requirements for the regulation of immune responses. Specifically, the evolutionary emergence of the ccr9/ccl25 receptor/ligand pair coincides with that of the thymus, whereas that of the cxcr4/cxcl12 pair antedates this.

### Control of Chemokine Expression in Thymic Epithelium

Next, we examined whether the gene encoding the novel chemokine ccl25 became integrated into an existing genetic network or whether a new network was required to regulate its expression. Of several chemokines in medaka, only *ccl25a* is expressed in the thymic rudiment (Figure 4A). Given that thymic epithelial cells in *Foxn1*-deficient mice lack the expression of *Ccl25* (Bleul and Boehm, 2000), we hypothesized that *Foxn1* might be genetically upstream of this chemokine. This was shown to be the case, using the transgenic HSE::*foxn1* and HSE::*foxn1*-enRD lines. Overexpression of wild-type *Foxn1* induced expression of *ccl25a*, whereas a dominant-negative version of *Foxn1* abolished it (Figure 4B). The expression of the paralogous gene *ccl25b*, which is not expressed in the thymus, remains unchanged under these conditions, providing an internal control for the specificity of the transcription factor function (Figure 4B). The coincident evolution of *foxn1* and *ccl25* in cartilaginous fishes and their functional interaction indicate that the functions of *foxn1* expanded and that the *ccl25a* gene is an evolutionarily novel target in thymic stromal cells.

### Control of Chemokine Receptor Expression in Lymphocytes

To identify potential regulators for *ccr9*, encoding the receptor for ccl25, we considered transcription factors that are highly expressed in thymocyte populations encompassing thymic immigrants (Rothenberg et al., 2008) that are known to express high levels of *Ccr9* (Benz and Bleul, 2005). This analysis suggested *Bcl11b* as a potential candidate. Indeed, it has been previously shown that lack of *Bcl11b* in mice results in severe defects in T cell development (Wakabayashi et al., 2003); *Bcl11b* is also expressed in the earliest identifiable thymocyte progenitors (I.R. and T.B., unpublished data). Furthermore, phylogenetic analysis of *Bcl11*-like genes (Figure S7A) suggests that, while homologs of *Bcl11a* are already present in the genomes of amphioxus and lamprey (and expressed in lamprey leukocytes, Figure 3A), a *Bcl11b* homolog first appears in cartilaginous fishes. In medaka (*O. latipes*) and zebrafish (*D. rerio*), only one of the two *bcl11b* homologs (*bcl11b.2*) is expressed in the developing thymus (Figure 4C). Overexpression of mouse *Bcl11b* under the control of a heat-shock promoter led to a dramatic increase in *ccr9* expression in the embryos (Figure 4D). Because a dominant-negative version of *Bcl11b* is not available, we examined *bcl11b.2* morphants for the expression of *ccr9*, and *rag1* as a general marker for differentiating thymocytes. The expression of *ccr9* in thymocytes of bcl11b.2 morphants is dramatically reduced (Figure 4E) in both species, indicating that in this context, *bcl11b* functions as an activator (Cismasiu et al., 2006) rather than a repressor (Cismasiu et al., 2005). Expression of *rag1* is unchanged, indicating that the number of thymocytes per se is not severely affected in these morphants and controlling for a possible effect of *Bcl11b* on T cell survival (Albu et al., 2007). No other abnormalities were detected in *bcl11b.2* morphants (Figure S7B). The coincident evolution of *bcl11b* and *ccr9* genes in cartilaginous fishes and their functional interaction indicate that they are part of an evolutionarily novel genetic network in the lymphocytes of jawed vertebrates.

### Functional Analysis of Progenitor Homing to the Thymus in Bony Fish

On the basis of the above experiments, we suggest that the common ancestor of vertebrates already possessed a primordial version of the future thymic microenvironment in the gill epithelium characterized by expression of orthologs of *foxn4L* and *dllb*, and that its lymphocytes expressed the genes for chemokine receptor *cxcr4* and the two *notch* receptors (Figure 5A). This raised the possibility that, in cartilaginous fishes, the genes encoding the novel *ccr9* chemokine receptor and the corresponding *ccl25* chemokine might have provided the essential cues for directing lymphocyte progenitors to the thymus anlage, enabling subsequent notch-delta interactions and eventually thymopoiesis. Moreover, since our expression survey in medaka embryos indicated that *cxcl12a* is expressed in the cells encapsulating the thymic epithelium (Figure 5B), which is consistent with possible functions in attracting lymphocyte progenitors to and/or retaining them near the thymic primordium, we examined a possible synergistic effect of *ccl25a* and *cxcl12a*. Knockdown of *ccl25a* alone or of both *ccl25* genes and/or *ccr9* genes specifically affected thymus homing, but did not abolish it (Figures 5C and S8A–S8C). As expected, generalized overexpression of *ccl25a* led to the absence of *rag1*-expressing cells in the thymus and the dispersion of lymphoid progenitors throughout the entire embryos (Figure S8D). Interference solely with *cxcl12* and/or *cxcr4* functions did not affect thymopoiesis (Figures 5C and S9A), although it affected the migration of primordial germ cells (Figure S9B), as expected (Raz and Reichman-Fried, 2006). By contrast, simultaneous interference with *ccl25a* and *cxcl12a* specifically and completely blocked thymopoiesis (Figure 5C); *ccl25b* failed to synergize with *cxcl12* (Figure S9C). To exclude the possibility that the lack of *rag1*-positive cells in the thymus of medaka morphants was due to an effect on lymphoid progenitors per se, we sought to establish a system in which the presence and migration of lymphoid progenitors could be

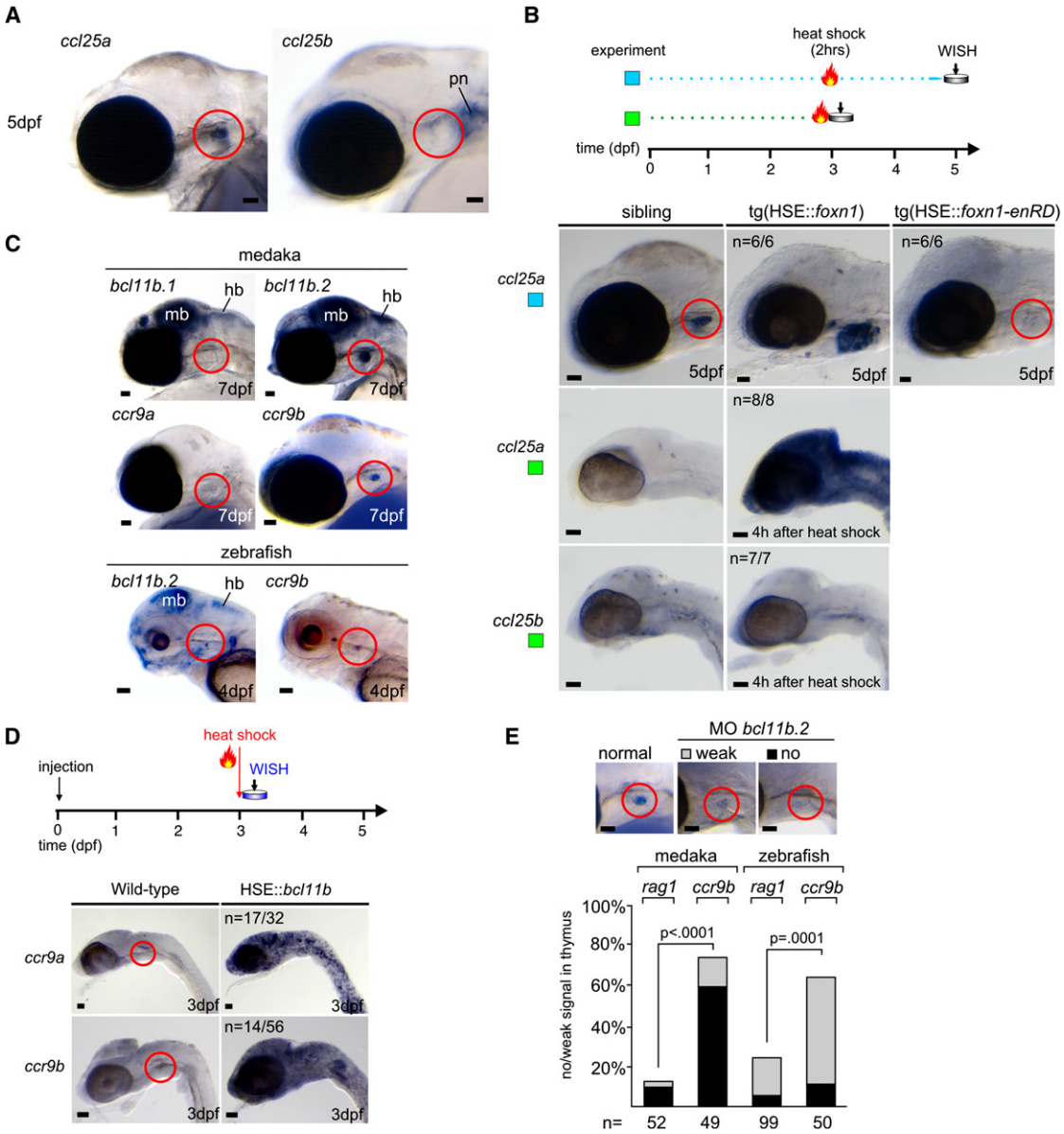



**Figure 4. Characterization of *foxn1*/*ccl25* and *bcl11b*/*ccr9* Networks**

(A and B) The gene encoding the ccl25a chemokine is a down-stream target of *foxn1*.

(A) Whole-mount RNA in situ hybridization indicates that only *ccl25a*, and not *ccl25b*, is expressed in the thymus (red circle) of medaka embryos. pn, pronephros. The gene encoding the chemokine ccl19 is not expressed in the thymus (data not shown).

(B) Induction of wild-type *foxn1* (HSE::*foxn1*) or dominant-negative *foxn1* (HSE::*foxn1*-enRD) in transgenic lines augments *ccl25a* expression in the thymus (red circle) or abolishes it, respectively. The number of embryos with the indicated expression patterns is shown. Note that *ccl25b* expression is unaffected by *foxn1* (bottom panel).

(C–E) The transcription factor bcl11b participates in the regulation of expression of the ccr9 chemokine receptor.

(C) Only one of the *bcl11b* homologs of medaka and zebrafish is expressed in the thymus as detected by whole-mount RNA in situ hybridization (*bcl11a.1* and *bcl11a.2* are not expressed in the thymus; data not shown); both homologs of *ccr9* are expressed, but *ccr9b* shows stronger expression in the thymus (see Figure S5). bcl11b genes are coexpressed in the midbrain (mb) and hindbrain (hb).

(D) Transient misexpression of *Bcl11b* results in overexpression of *ccr9*. Mouse *Bcl11b* cDNA was cloned into the heat-shock vector shown in Figure 2B. The number of embryos with the indicated expression patterns is shown; position of the thymus is marked by red circle.

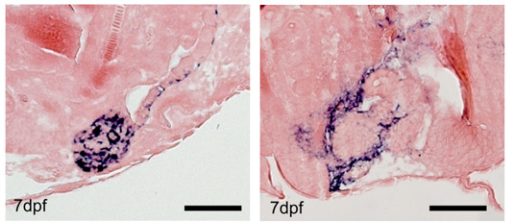
(E) Knockdown of *bcl11b.2* selectively abolishes *ccr9* expression in the thymus, while only mildly affecting *rag1* expression (morpholino concentration was 800 μM for medaka and 500 μM for zebrafish embryos); in embryos injected with a control morpholino, *rag1* expression levels are normal (data not shown; see also Figure 5C). Examples of expression patterns and their classification in medaka embryos are shown at the top. The position of the thymus is highlighted in red.

Scale bars represent 50 μm.

A

| Species | Morphological character: Lymphocytes | Morphological character: Thymus | Gene content: *Foxn* | *Delta-like* | *Cxcl12* | *Ccl25* | *Bcl11* | *Notch* | *Cxcr4* | *Ccr9* |
|---|---|---|---|---|---|---|---|---|---|---|
| *Mus musculus* | + | + | 1,4 | 1,3,4 | + | + | a,b | 1,2,3,4 | + | + |
| *Oryzias latipes* | + | + | 1,4 | 1a,1b, 3 4a,4b | a,b | a,b | a.1,a.2 b.1,b.2 | 1a,1b 2,3 | a,b | a,b |
| *Callorhinchus milii* | + | + | 1,4 | 1a,1b,1c | a,b | + | a,b | 1,1L,3 | + | + |
| *Petromyzon marinus* | + | - | 4L,4 | a,b,c | + | - | a | a,b | + | - |
| *common vertebrate ancestor* | + | - | 4',4 | 1',1 | + | - | a | 1',1 | + | - |
| *Branchiostoma floridae* | ? | - | 4a,4b | 1 | - | - | a | 1 | - | - |

B

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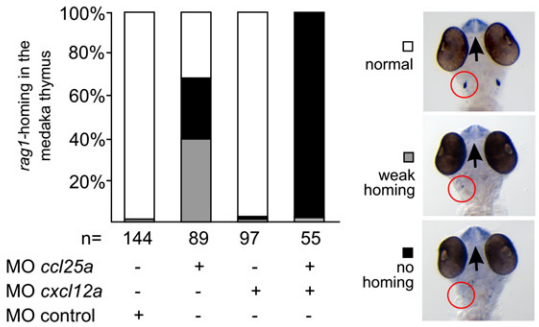


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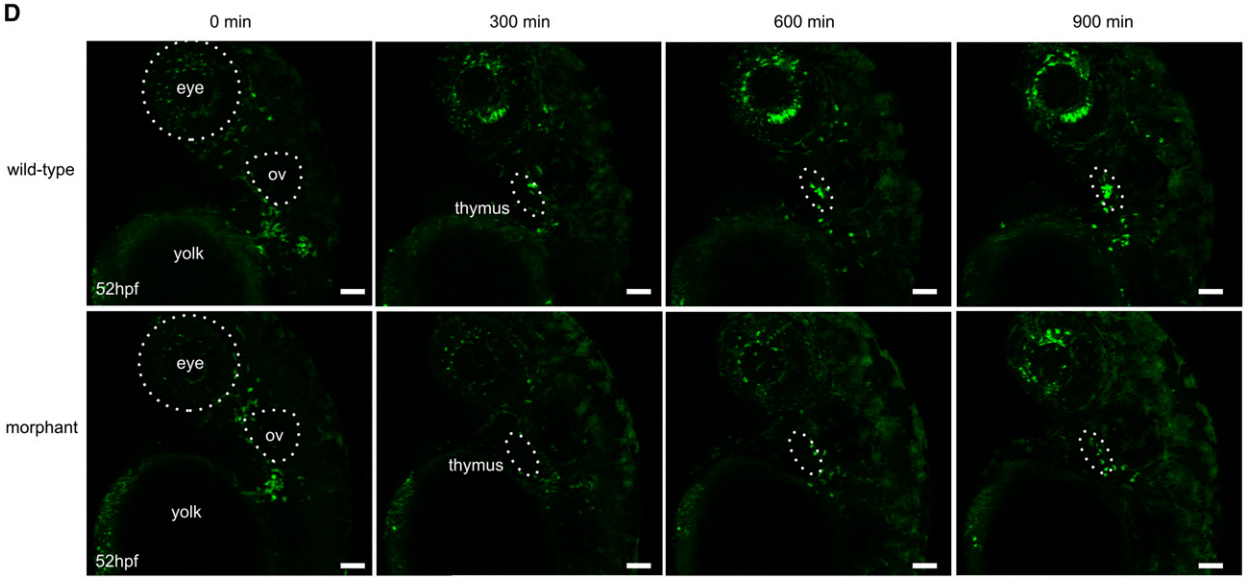


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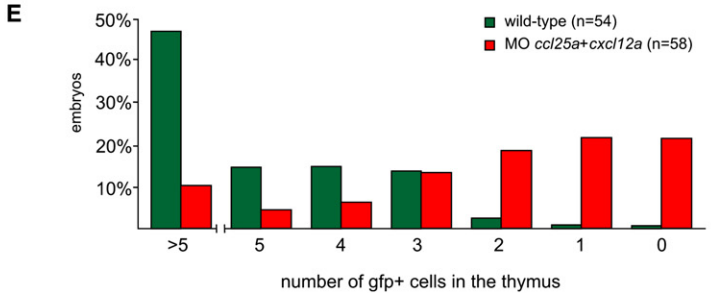

conveniently examined by time-lapse microscopy. To this end, we established a transgenic zebrafish line that expresses GFP under the control of the transcriptional regulatory elements of the *ikaros* gene, a marker of lymphoid progenitor cells (Schorpp et al., 2006) (Figure S10). Thymus homing begins at 54–56 hours postfertilization (hpf) (Figure 5D; Movie S1), in agreement with earlier studies with a different reporter system (Kissa et al., 2008). In zebrafish *ccl25a*/*cxcl12* morphants, lymphoid progenitors are generated in normal numbers; they are mobile, but their homing to the thymus is specifically impaired (Figures 5D and 5E; Movie S2), in agreement with the findings in medaka morphants. Collectively, these findings suggest that thymus colonization requires the cooperation of evolutionarily ancient (*cxcr4*/*cxcl12*) and new (*ccr9*/*ccl25*) chemokine/chemokine receptor pairs and might explain why thymus homing is not abolished in mice deficient for either Cxcl12/Cxcr4 or Ccl25/Ccr9 (Benz and Bleul, 2005; Uehara et al., 2002; Wurbel et al., 2001, 2007).

Finally, we examined the role of *foxn1* in thymopoiesis. First, we examined the fate of lymphoid progenitors in transgenic medaka, using *ccr9a* as a general marker of lymphoid progenitors and *rag1* as a marker of thymocytes. In HSE::*foxn1*-enRD lines, *ccr9a*-expressing lymphoid precursors are lacking in the thymus rudiment, but are instead found in its vicinity; hence, thymopoiesis fails as indicated by the absence of *rag1* expression (Figure 6A). This finding indicates that lack of *foxn1* affects thymopoiesis more severely than can be explained by lack of *ccl25a* expression alone (Figures 4B and 5C), presumably because it interferes with additional functions of the thymic epithelial microenvironment, including *dll4a* expression (Figure 2). In HSE::*foxn1* lines, *ccr9a*-expressing lymphoid progenitors are found dispersed in the embryos (Figure 6A), possibly owing to the generalized misexpression of *ccl25a* (Figure 4B) and subsequent disorientation of lymphoid progenitors (cf. Figure S8D). The lack of thymopoiesis under conditions of *foxn1* deficiency was confirmed by use of the zebrafish *ikaros::gfp* transgenic line (Figures 6B and 6C).

## DISCUSSION

We have used comparative genome analysis and coexpression studies in species occupying relevant positions on the phylogenetic tree and functional tests to trace the evolution of genetic networks controlling two key aspects of thymopoiesis, namely attraction of lymphocyte progenitors to the thymic anlage and specification of progenitors to the T cell lineage. Our data suggest that some of the key elements of the genetic networks of the pharyngeal epithelium and lymphocytes subsequently co-opted for thymopoiesis were pleisiomorphic features of the common vertebrate ancestor.

### Pleisiomorphic Features of Thymic Epithelium

The gill basket is a complex morphological structure, with numerous adaptations to facilitate exchange of oxygen and uptake of food in the same anatomical structure. In lamprey, the *foxn4L*/*dllb* expression domain occurs in the epipharyngeal ridge (Wallin, 1917) and appears to distinguish nonciliated from ciliated epithelial cell types in this structure; whether this correspondence is merely coincidental or indicative of a particular specification function awaits further study. *Delta*-like genes appear to be primordial targets of *Foxn4*-like genes, as indicated by coexpression of *Foxn4* and *Dll* in the cephalochordate amphioxus and of *foxn4L* and *dllb* in the lamprey; indeed, this function has been evolutionarily conserved in the thymic epithelium (this paper) and in other tissues (Chi et al., 2008; Li et al., 2005) of higher vertebrates. This suggests that the relevant *cis*-regulatory elements were maintained after the genome duplication in the ancestral cartilaginous fishes (Escriva et al., 2002). It is unknown whether orthologs of *Foxn4* or *Foxn1* are also involved in regulating the expression of notch receptors to achieve mutually exclusive expression of *notch* and *dll* genes. In any event, the regionalized coexpression of an ortholog of *Foxn1* and a *Dll*-homolog is compatible with the emergence of the thymus in the pharyngeal region of jawed vertebrates and provides an explanation for why the thymus arose at this position. The genome duplication accompanying the transition from the common vertebrate ancestor to cartilaginous fishes also resulted in the appearance of a novel chemokine gene, *ccl25*, presumably as a paralog of one of the primordial vertebrate *ccl* genes. Although the expression sites of primordial *ccl* genes are unknown, it is likely that this gene acquired a Foxn1-responsive *cis*-regulatory element to become a target of this transcription factor in jawed vertebrates. This modification enabled the prospective thymic anlage in the pharyngeal epithelium to attract lymphocytes to a particular kind of epithelial environment to which lymphocytes

**Figure 5. Functional Analysis of Thymus Colonization in Medaka Embryos**

(A) Gene content of chordate genomes and the presence of lymphocytes and thymus. The presence of the thymus (Pastoret et al., 1998) correlates with the emergence of *Foxn1*, *Ccl25*, *Bcl11b*, and *Ccr9* genes. See Figures S1, S3, and S4–S7 for details. The presence of bona fide lymphocytes in amphioxus is unclear (Ruppert, 1997). The information given for the common vertebrate ancestor is hypothetical; paralogs are indicated by apostrophes.

(B) Expression of chemokine genes *ccl25a* and *cxcl12a* in the thymic rudiment of medaka embryos as detected by RNA in situ hybridization on tissue sections. *ccl25a*, but not *ccl25b* (see Figure 4A), is expressed in the epithelial rudiment of the thymus; *cxcl12a*, but not *cxcl12b* (data not shown), is expressed in the connective tissue encapsulating it. The chemokine receptor genes *ccr7* and *cxcr7* are not expressed in the thymus (data not shown). Scale bars represent 50μm.

(C) Synergism of *ccl25a* and *cxcl12a* in the regulation of thymus homing in medaka embryos; double morphants lack *rag1*-expressing cells in the thymus but are otherwise normal (as examined by expression of dlx2 and *l-plastin*; data not shown). Morpholino concentrations were 200 μM each, with the exception of the control morpholino, which was 1 mM. Examples of WISH results for classification of embryos are shown in the right panel.

(D) Time-lapse recording of thymus homing in zebrafish wild-type (upper panels) and ccl25a/cxcl12 morphants (at 100 μM each; lower panels). Still photographs taken at various time points (0 min corresponds to 52 hpf; before the onset of homing) highlighting the homing of *ikaros* expressing lymphoid progenitors to the thymus in zebrafish embryos transgenic for *ikaros::gfp*. Frames are taken from Movie S1 (wild-type) and Movie S2 (morphants). Note that thymus homing eventually resumes in morphants, owing to the dilution of morpholinos in dividing cells. Scale bars represent 50 μm.

(E) Thymus homing in wild-type and ccl25a/cxcl12a morphant (at 100 μM each) zebrafish embryos. The percentages of embryos with the indicated number of cells in the thymus are shown for the 63 hpf time point.

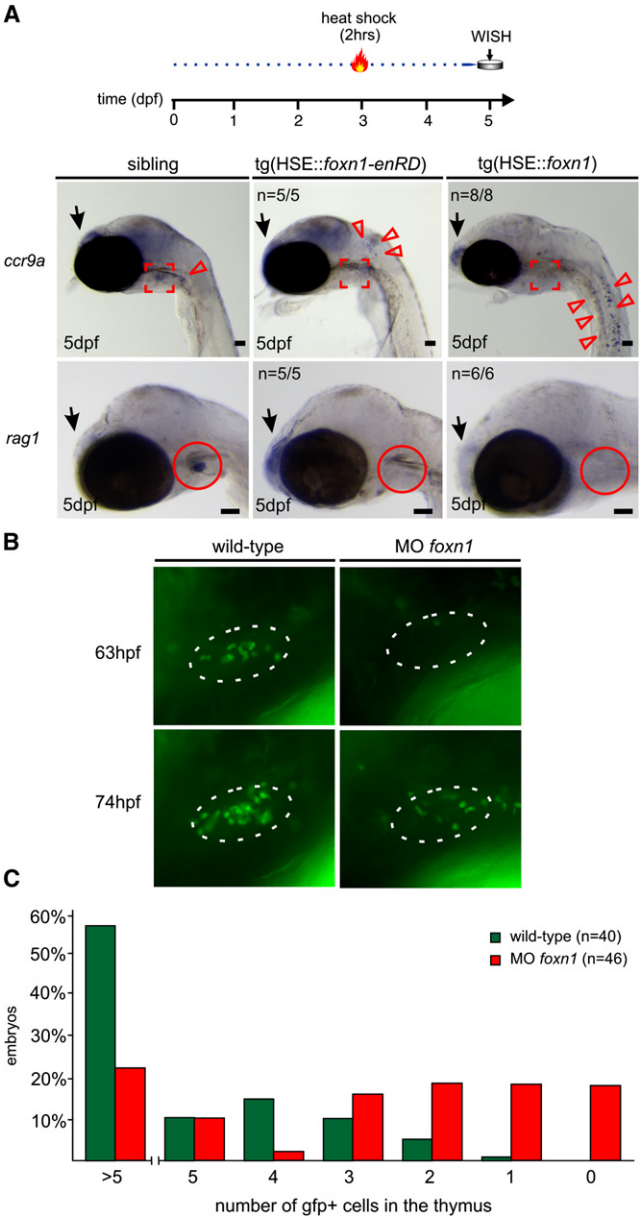


**Figure 6. Evolutionarily Conserved Function of *foxn1* in Thymopoiesis**

(A) Interference with *foxn1* function by transient overexpression abolishes thymopoiesis in transgenic medaka embryos. Expression of a dominant-negative form of *foxn1* leads to accumulation of *ccr9a*-expressing lymphoid progenitors (red arrowheads) in the vicinity of the thymus (marked in red), whereas overexpression of wild-type *foxn1* leads to their dispersal in the entire embyro (top panels). Thymopoiesis fails as indicated by the lack of *rag1*-expressing cells (bottom panels). The number of embryos with the indicated phenotypes is shown. Scale bars represent 50 μm.

(B) Impaired thymopoiesis in zebrafish *foxn1* morphants. Photographs were taken at the indicated time points from the thymus region of wild-type (left panels) or morphant (at 200 μM each; right panel) embryos of the *ikaros::gfp* transgenic line. Note that thymus homing eventually resumes in morphants owing to the dilution of morpholinos in dividing cells.

(C) Thymus homing in wild-type and *foxn1* morphant zebrafish embryos. The percentages of embryos with the indicated number of cells in the thymus are shown for the 63 hpf time point.

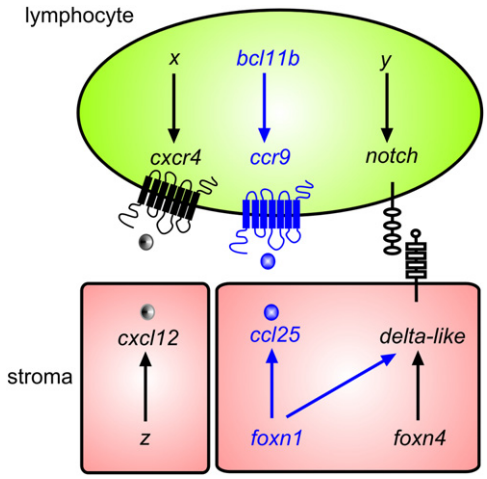


**Figure 7. Structure of Evolutionarily Ancient and Novel Genetic Networks Regulating Thymopoiesis**

Networks emerging in jawed vertebrates are indicated in blue.

of the common vertebrate ancestor and lamprey had no access. Because delta-like ligands were already expressed at this site, this simple elaboration of the genetic network controlled by Foxn1 now enabled the two cardinal functions of the thymic microenvironment, namely attraction and specification of lymphocyte progenitors through their notch receptors (Figure 7).

## Pleisiomorphic Features of Lymphocytes

It is conceivable that expression of *cxcr4* and *notch* in lymphocytes was already established before the emergence of jawed vertebrates. In the common vertebrate ancestor (and perhaps also in the extant jawless fishes), these genes might have been involved in guiding the differentiation of lymphocytes; indeed, in the mouse, development of T and B cells is differentially regulated by Notch signaling (Tanigaki and Honjo, 2007). Notch ligands required for specification were presumably supplied in general hematopoietic tissues, such as the equivalent of the lamprey typhlosole; in addition, the cxcr4/cxcl12 chemokine receptor/ligand pair might have been involved in regulating the precise spatial distribution of lymphoid progenitors in hematopoietic tissues. In cartilaginous fishes, the gene encoding the chemokine receptor ccr9 possibly arose from one of the primordial *ccr* genes of the basal vertebrate; likewise, the gene encoding the bcl11b transcription factor appears to be a paralog of the ancient *bcl11a* gene. It is unclear whether bcl11a already controlled the expression of a primordial *ccr* gene in lymphocytes; if not, a bcl11b-responsive element must have been added to the transcriptional regulatory region of the *ccr9* gene. The fact that *bcl11a* is expressed in lamprey lymphocytes suggests that its transcriptional control elements already directed expression in this cell type in the common vertebrate ancestor before the emergence of jawed vertebrates and that they were probably maintained in the duplicated *bcl11b* gene. As a consequence of these adaptations, lymphocytes of cartilaginous fishes now expressed—in addition to the ancient *cxcr4* chemokine receptor and *notch* genes—ccr9 as a novel receptor for the ccl25 chemokine (Figure 7), allowing them to

respond to the signals emanating from a localized source in the pharynx. The identity of the transcriptional regulator(s) of *cxcr4*, *bcl11b*, and *notch* genes in lymphocytes is not known.

### Further Components of Genetic Networks Controlling the Development of Lymphocytes

Although not directly addressed in this paper, it is of interest to consider the evolution of other components of the genetic network controlling thymocyte differentiation. Detailed functional analyses in mice indicate that several secreted factors play an important role in the development of lymphocytes. One of the most important cytokines is interleukin 7 (Il-7); in mice, it affects both T and B cell development (reviewed in Kang and Der, 2004). This suggests that Il-7 might be an ancient factor required for lymphocyte precursor maturation and survival and that it might have emerged in the common vertebrate ancestor, perhaps concurrently with lymphocytes. Although the evolutionary history of genes encoding Il-7 and its receptor complex (Il7r$\alpha$ and $\gamma_c$) and their expression domains has not yet been analyzed in detail, it is perhaps worth noting that intracellular signaling from an evolutionarily novel receptor occurs through the evolutionarily ancient Jak/Stat pathway (Kang and Der, 2004). In mice, lymphocyte development additionally requires the function of two structurally related receptors, c-kit for T cells (Di Santo and Rodewald, 1998) and Flt-3 for B cells (Singh et al., 2005); it appears that their cognate ligands, Scf and Flt3-L, respectively, are required before the Il-7-dependent phase of lymphocyte development and are thus perhaps involved in maintaining progenitors biased toward the T and B cell lineages, respectively. Hence, it is possible that their function in lymphocytes was realized at the same time as the emergence of different lymphocyte lineages in cartilaginous fishes. The fact that the c-kit/Scf receptor/ligand pair is important in a multitude of progenitor cell types (Besmer et al., 1993) suggests its ancient origin (perhaps before the emergence of vertebrates); it may also have given rise, through gene duplications, to the homologous Flt3/Flt3L receptor/ligand pair (Rousset et al., 1995; Savvides et al., 2000). It also follows that the role of c-kit/Scf in the T cell lineage is probably a derived function; hence, the evolutionary history of c-kit/Scf and Flt3/Flt3L might illuminate the so far unresolved question of whether the primordial lymphocyte was more T or B like (Davis and Bjorkman, 1988; Kawamoto, 2006; Boehm and Bleul, 2007) or whether they emerged simultaneously.

### Selective Advantage Associated with Expansion of Genetic Networks

To date, we can only speculate about the selective advantage for basal jawed vertebrates associated with the expansion of the primordial genetic networks later co-opted for the elaboration of thymopoiesis. It is possible that the emergence of different lymphocyte lineages might have coincided with, and perhaps even facilitated, the acquisition, by lateral gene transfer, of the VDJ recombination machinery (Schatz, 2004). In this context, it is worth noting that structural arguments support the idea that the T cell receptor (TCR) evolved as a dimeric primordial MHC receptor (either as a homodimer or a heterodimer containing a homolog of the pre-T$\alpha$ chain) and that it later gave rise to the immunoglobulins (Davis and Bjorkman, 1988). The elaboration of a new kind of cytotoxic lymphocyte, distinguished by the expression of somatically diversifying antigen receptors, might have at least partially satisfied increased demands for immune defense in basal vertebrates (Cooper and Alder, 2006). Cell-cell interactions are not only required for fate determination, but also for the quality-control process (Boehm, 2006a) required to tame the intrinsic propensity for self-reactivity of VDJ-type antigen receptors bearing essentially random binding sites. In the T cell lineage, this depends on the interaction of the TCR with peptide/MHC complexes that are expressed on the thymic epithelium; we have discussed elsewhere how an ancient form of the MHC-based peptide presentation system might have been co-opted for this somatic quality control (Boehm, 2006b). Cell nonautonomous requirements such as those described above provide a conceptual framework to understand the selective value of exposing lymphocytes to the primordial thymic epithelium.

In conclusion, we have provided an outline for the evolutionary origins of two major aspects of thymopoiesis, i.e., attraction and specification of lymphoid progenitors. We anticipate that our integrated approach could also be used to examine other aspects of T cell development and the adaptive immune system of jawed vertebrates.

## EXPERIMENTAL PROCEDURES

### Phylogenetic Analyses

Phylogenetic relationships were deduced after alignment of protein sequences with programs implemented in the DNAstar suite (http://www.dnastar.com); for information on sequences, see the Supplemental Data.

### Animals

European brook lampreys (*L. planeri*) were sampled in the Freiburg area with permission of the local authorities, small spotted catsharks (*S. canicula*) were kindly donated by Dr. T. Jermann (Zoological Garden of Basel, Switzerland), the cab-strain of wild-type medaka (*O. latipes*) was kindly provided by J. Wittbrodt (EMBL Heidelberg, Germany), the TLEK strain of zebrafish (*D. rerio*) is kept at this institute, and white leghorn chicken (*G. gallus*) embryos were a kind gift of Dirk Junghans (this institute). Medaka embryos were staged according to Iwamatsu (Iwamatsu, 2004).

### *D. rerio* Transgenic Line

The BAC construct used to generate the *ikaros::gfp* reporter line is schematically depicted in Figure S10A (see the Supplemental Data for further details). Comparative analysis of *ikaros* mRNA in situ hybridization and GFP expression reveals compete concordance (Figure S10B).

### *O. latipes* Stable Transgenic Lines

Two transgenic lines were generated for this study with the constructs schematically depicted in Figure 2 (see the Supplemental Data for further details).

### *O. latipes* Transient Misexpression Studies

Medaka full-length *ccl25a* gene (equivalent to nt sequence of Ensembl-ID ENSORLT00000022583) or mouse *Bcl11b* (equivalent to nt sequence in accession number BC019503) was cloned into the heat-inducible pSGH2 vector (Bajoghli et al., 2004).

### Analysis of Medaka and Zebrafish Morphants

Phosphorodiamidate morpholino oligonucleotides (abbreviated here as morpholinos) were manufactured by Gene Tools (Philomath, OR) (see Table S2). For injection into medaka embryos, morpholinos were dissolved in 1× Yamamoto's Ringer solution (Yamamoto, 1975) at the indicated concentrations and injected into blastomeres of medaka embryos as described (Carl et al., 2002).

For zebrafish morphants, morpholinos were dissolved in 1× Danieau buffer (Westerfield, 1994) at the indicated concentrations and injected into the yolk of zebrafish embryos at the one- or two-cell stage.

### In Situ Hybridization Analysis

Whole-mount in situ hybridization (WISH) in medaka and zebrafish was performed with digoxigenin-labeled RNA riboprobes as described (Aghaallaei et al., 2007; Schorpp et al., 2006). Whole-mount RNA in situ analysis in amphioxus embryos was done as described (Holland et al., 1996). Probes are listed in Table S3.

### RT-PCR Analysis

Leukocytes from lamprey kidney tissue were sorted according to their size (FSC) and granularity (SSC) (see below) and directly lysed in Trizol reagent (Sigma); other tissues were pulverized in liquid nitrogen prior to Trizol lysis. Total RNAs were purified by chloroform extraction and precipitation with isopropanol. RNA samples were treated with RNase-free DNase (Roche) before first-strand cDNA synthesis with random hexamer primers and SuperscriptII (Invitrogen); RT-negative reactions were performed under the same conditions, but in the absence of the SuperscriptII enzyme. The first-strand cDNA was directly used as template in PCR reactions. To reveal expression and to distinguish the two *notch* genes in lamprey, a second round of PCRs was used; *vlr-b* expression was detected by nested PCR reactions. Primers are listed in Table S1.

### Flow Cytometry and Cell Sorting

*L. planeri* larvae (10–14 cm long) were anaesthetized with 150 mg/l MS222 (Sigma) and decapitated behind the last gill opening. Blood was immediately diluted with 0.57 × PBS/30 mM EDTA, and blood cells were pelleted (300 g for 5 min at 4°C), resuspended in FACS buffer (5 mM EDTA and 10 U/ml Heparin in 1 × PBS) and sorted on a FACSAria Cell Sorting System (BD Biosciences) according to their size (FSC) and granularity (SSC).

### Confocal and Time-Lapse Microscopy of *ikaros::gfp* Embryos

At 52 hpf, embryos were anesthetized with tricaine methanesulfonate, immobilized in 0.8% low-melting agarose, covered with embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CacCl_2$, 0.33 mM $MgSO_4$) and kept in a heating chamber at 28°C. For short-term imaging, the embryos were anesthetized and immobilized in 3% methylcellulose. Fluorescence microscopy was performed with Zeiss Imager.Z1, confocal time-lapse imaging (one image every 2.5min) was done with a Zeiss LSM510 Meta NLO, and pinhole was set to 1.14 airy units. Movies were taken with Zeiss LSM4.2 software, single frames were generated as projections of a stack of 12 planes with 5 μm distance, and schematics were generated with Adobe Premiere 5.5 software.

### Statistical Methods

A Chi-square test of association was used in the experiment shown in Figure 4E.

## ACCESSION NUMBERS

Sequences were deposited with GenBank under accession numbers FJ176201, FJ176202, and FJ187748–FJ187756.

## SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, ten figures, and three tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00442-5.

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