Sequential organogenesis sets two parallel sensory lines in medaka

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ABSTRACT

Animal organs are typically formed during embryogenesis by following one specific developmental programme. Here, we report that neuromast organs are generated by two distinct and sequential programmes that result in parallel sensory lines in medaka embryos. A ventral posterior lateral line (pLL) is composed of neuromasts deposited by collectively migrating cells whereas a midline pLL is formed by individually migrating cells. Despite the variable number of neuromasts among embryos, the sequential programmes that we describe here fix an invariable ratio between ventral and midline neuromasts. Mechanistically, we show that the formation of both types of neuromasts depends on the chemokine receptor genes cxcr4b and cxcr7b, illustrating how common molecules can mediate different morphogenetic processes. Altogether, we reveal a self-organising feature of the lateral line system that ensures a proper distribution of sensory organs along the body axis.

KEY WORDS: Organogenesis, Lateral line, Cxcr4b, Cxcr7, Eya1, Neuromast

INTRODUCTION

The diversity of body forms across the animal kingdom is astonishing. Even within taxa, species show major differences in body size and relative shape. Teleost fish are arguably the most extreme example of such diversity among vertebrates (Coombs et al., 2014, 1988). The arrangement of neuromasts on the head, the range from a seahorse to an eel, a pufferfish, a tuna and a zebrafish. When reviewing all these diverse forms, we wondered how peripheral sensory systems could adapt to such dissimilar body shapes. Here, we tackle this question by focusing on the embryonic formation of the posterior lateral line (pLL) system in medaka embryos (Oryzias latipes).

The lateral line system is composed of neuromasts, differentiated organs containing dozens of cells that sense the direction of flow and relay this information to the brain (Ghysen and Dambly-Chaudière, 2007). The arrangement of neuromasts on the head, main body and tail is species specific (Sapêde et al., 2002) and relates to the body shape of the fish (Coombs et al., 2014, 1988). Most of our knowledge on lateral line development comes from studies on the pLL of zebrafish; several research groups have contributed molecular, genetic and physiological approaches that have deepened our understanding on the morphogenesis, homeostasis and regeneration of the system (Aman and Piotrowski, 2008; David et al., 2002; Grant et al., 2005; Haas and Gilmour, 2006; Hernández et al., 2006; Lecaudy et al., 2008; López-Schier and Hudspeth, 2005, 2006; Lush and Piotrowski, 2014; Ma and Raible, 2009; McGraw et al., 2014; Nechiporuk and Raible, 2008; Pichon and Ghysen, 2004; Sapêde et al., 2002; Sánchez et al., 2016). The formation of the pLL starts with a group of cells (a primordium) that delaminates near the otic vesicle from the lateral line placode and migrates posteriorly along the horizontal myoseptum (Ghysen and Dambly-Chaudière, 2007; Kimmel et al., 1995). A first primordium (primI) deposits five to eight pre-formed neuromasts from its rear edge (Ghysen and Dambly-Chaudière, 2007; Lecaudy et al., 2008; Nechiporuk and Raible, 2008) at regular intervals, which are connected by so-called inter-neuromast cells (ICs). This is followed by a second primordium (primII) that runs along the same path to deposit additional neuromasts between the ones previously generated by primI (Ghysen and Dambly-Chaudière, 2007; Ledent, 2002; Sapêde et al., 2002). Both sets of neuromasts migrate ventrally (Ghysen and Dambly-Chaudière, 2007; Ledent, 2002; Whitfield, 2005), and during the first post-embryonic days new neuromasts are formed by proliferation of the ICs (Grant et al., 2005; López-Schier and Hudspeth, 2005; Lush and Piotrowski, 2014; Whitfield, 2005) to complete a pLL system that displays roughly one neuromast per somite boundary. There are additional primordia that generate the dorsal pLLs (Ghysen and Dambly-Chaudière, 2007; Nuñez et al., 2009; Sapêde et al., 2002), and these and other observations have contributed to the notion that the different pLLs in fish are each generated by different primordia.

The anterior-to-posterior migration of primI depends on Cxcl12a/Cxcr4b/Cxcr7b signalling (David et al., 2002; Haas and Gilmour, 2006; Valentin et al., 2007); the migrating primordium has been shown to generate its own Cxcl12a gradient via the opposing roles of the two chemokine receptors Cxcr4b and Cxcr7b (Donà et al., 2006; Valentin et al., 2007). Interestingly, rescue by wild-type cells occurs if these are located at the leading edge of the primordium, which nevertheless deposits mutant cells at the rear to generate all the neuromasts of the pLL system (Haas and Gilmour, 2006). Therefore, primordium migration can be achieved with just a few cells that receive and process the ligand information.

Our current understanding of how the pLL develops in other fish is limited due to the lack of proper genetic tools to characterise the morphogenetic process dynamically. In the context of the diversity of body shapes among teleosts, the ‘one-primordium one-lateral-line’ concept would imply the existence of more primordia in fish.
displaying more lateral lines. Interestingly, medaka embryos are larger than zebrafish embryos and display an extra lateral line at the time of hatching. We therefore decided to expand the analysis of the pLL system to medaka, which offers extensive transgenic and experimental embryology approaches (Kirchmaier et al., 2015), to address how diverse distributions of sensory organs arise during development.

We generated a transgenic line in medaka that specifically labels neuromasts using the eyes-absent-1 (eya1) promoter to follow the dynamic formation of the pLL in vivo. We observed that neuromasts of two parallel pLLs in medaka are formed in a sequential manner by cells coming from a common primordium. The first set of organs are deposited from the rear of a migrating primordium and shortly after start to migrate ventrally forming the ventral pLL. A second set of neuromasts is formed by ICs and cells that escape from previously formed organs, organise into a cluster and migrate dorsally to form the midline pLL. We demonstrate that Cxcr4b and Cxcr7 are used iteratively: initially to drive the migration of the primordium and afterwards in individual cell migration during secondary organ formation. Finally, we show that this mode of organogenesis, whereby already generated neuromasts can produce second generation organs, is controlled within the lateral line and propose that this could facilitate the adaptation of the sensory system to the variety of body shapes found among teleost fish.

RESULTS
Alternated organ distribution between two parallel pLLs
At the end of embryogenesis, the pLL system of medaka exhibits two parallel sets of mature neuromasts, arranged ventrally and on the midline (Fig. 1A-C) (Ishikawa, 1994; Sapede et al., 2002; Wada et al., 2008; Yasuoka et al., 2004). This differs from the embryonic patterns of other teleost fish such as zebrafish or Astyanax, in which most neuromasts are found along one line (Fig. 1A) (Sapede et al., 2002). The additional pLL in medaka is believed to originate from an independent primordium, a speculation that has not yet been formally tested. When quantifying the neuromasts in each pLL, we noticed that ventral and midline neuromasts are arranged in...
staggered rows, where each neuromast in the midline pLL (mpLL) is surrounded by an anterior and a posterior neuromast in the ventral pLL (vpLL) (99.2%; n=141 neuromasts, n=22 pLLs) (Fig. 1B,C). The two-to-three most anterior and the two-to-three most posterior neuromasts of the ventral pLL do not have a counterpart in the midline (22/22 pLL and 21/22 pLL, respectively) (Fig. 1C). The alternated pattern of neuromasts in the ventral and midline pLL suggests a coordinated, rather than a complementary, organogenesis between the two pLLs.

To follow lateral line formation in medaka in vivo, we created transgenic lines in which the promoter of the medaka eyes-absent 1 gene (eya1) drives the expression of different fluorescent proteins. Previous studies indicated that eya1 is a bona fide marker for the lateral line in medaka, as eya1 mRNA in situ hybridisation labels neuromasts during embryonic and early post-embryonic stages (Fig. 1C) (Wada et al., 2008). Our transgenic lines recapitulate medaka eya1 expression (Fig. 1D) and allow in vivo visualisation of neuromasts during embryonic, juvenile and adult stages (Fig. 1D-I; Fig. S1). The alternated distribution of neuromasts in Tg(Eya1:EGFP) embryos was identical to non-transgenic controls (99%; n=197 midline neuromasts, n=32 pLLs), with around ten neuromasts in the ventral pLL (10.1±0.9; n=126 pLLs) and between six and seven in the midline pLL (6.6±0.8; n=126 pLLs). All mature neuromasts from the different post-embryonic lateral lines were labelled by Tg(Eya1:EGFP) and by Tg(Eya1:2H2B-EGFP) as indicated by co-staining with specific vital dyes (DiAsp, DAPI; Fig S1) for the differentiated neurons of the organ (n>100 neuromasts, n>10 larvae for each transgenic line). In addition, our transgenic lines allow the visualisation of immature neuromasts that have not yet generated differentiated cells (Fig. S1). Therefore, Tg(Eya1:EGFP) recapitulates the endogenous eya1 expression and allows neuromast formation to be visualised in vivo.

**Sequential formation of vpLL and mpLL neuromasts from a common primordium**

Based on abundant data from zebrafish studies in which multiple migrating primordia have been shown to generate different pLLs (Glysen and Dambly-Chaudière, 2007; Ledent, 2002; Nuñez et al., 2009; Sapêde et al., 2002), it was largely assumed that each pLL in medaka derived from an independent primordium during embryonic development (Sapêde et al., 2002). We used Tg(Eya1:EGFP) to follow embryonic pLL morphogenesis dynamically, and observed a primordium that delaminates near the otic vesicle from the lateral line placode at stage 26 and migrates posteriorly (Fig. 2A-B) along the myoseptum to reach the tail about three days later (Fig. 2C). Interestingly, this primordium generates approximately 12-14 neuromasts that are deposited along the myoseptum – the number of neuromasts later found at the ventral pLL (Fig. 2C). Iterative observation of individual Tg(Eya1:EGFP) embryos indicated that the neuromasts produced by the primordium migrate ventrally and form the ventral pLL (Fig. 2D-E’; Movie 1). Intriguingly, we could never detect a second primordium as reported in zebrafish and, yet, we could observe the appearance of about seven midline pLL neuromasts distributed along the myoseptum on the following days (Fig. 3A; Fig. 1C,D). These results suggest either the presence of a second eya1-negative primordium, or different mechanisms for generating neuromasts in the midline and in the ventral pLLs.

To investigate the origin of the midline pLL neuromasts, we used two-photon laser ablations to remove the primordium just after the first neuromast was deposited (Fig. 3B, scheme). Ablation of primI in early zebrafish results in the absence of the primI-produced neuromasts, but it does not affect the migration of primII nor the generation of primII-derived neuromasts (López-Schier et al., 2004). After ablation, Tg(Eya1:EGFP) embryos were developed until juvenile stages. We observed that although the control side displayed complete ventral and midline pLLs, early primordium ablation on the experimental side resulted in the absence of all neuromasts both on the ventral and the midline pLLs (n=4 juveniles analysed; control side: 9.5±1.3 ventral neuromasts and 5.5±1 midline neuromasts; experimental side: 1 ventral neuromast and no midline neuromasts) (Fig. 3A,B). Later primordium removal resulted in the absence of neuromasts posterior to the ablation site in the ventral and the midline pLL (n=4) (Fig. 3C, scheme) but no evident phenotype anterior to the ablation site (Fig. 3C). These results strongly suggest that a single primordium is responsible for the generation of the two parallel pLLs in medaka, ruling out the one-primordium one-lateral-line concept.

If the two parallel pLLs come from the same primordium, when do they split apart? And when do they reach their final destination?
By following a 4D approach on Tg(Eya1:EGFP), we observed that ventral migration of the neuromasts occurs soon after the primordium deposits each organ (Fig. 2D-D″; Movie 1). Therefore, the most anterior neuromasts start their ventral migration while the primordium is still on its way towards the tail (Fig. 2D-E′; Movie 1). After the initiation of ventral migration, we detected the appearance of new neuromasts (hereafter termed secondary neuromasts) between the ones that were originally deposited by the primordium (hereafter termed primary neuromasts) (Fig. 4A,B). The temporal appearance of secondary neuromasts follows the same anterior-posterior dynamics observed in the primary neuromasts. Secondary neuromasts are formed between all pairs of consecutive primary neuromasts (Fig. 4A,C), excluding the first two and last pairs. This alternating pattern resembles our previous observations comparing the ventral and the midline pLLs and suggests that secondary neuromasts are forming the midline pLL. Indeed, secondary neuromasts start migrating dorsally soon after being formed (Fig. 4B; Movie 2), and reach their final position just dorsal to the myoseptum (Fig. 4C). Overall, our results show that the two pLLs in medaka are formed in different manners and involve opposite migration. The ventral pLL is formed by neuromasts that are deposited in the midline by the primordium and then migrate ventrally. The midline pLL is formed by neuromasts that originate at the ventral part of the fish and migrate dorsally to the myoseptum (Fig. 4C).

**Individual cell migration initiates secondary neuromast formation**

The formation of secondary neuromasts could be driven by signals outside or within the pLL system. Empty inter-somitic spaces could function as a call for the generation of secondary organs (Núñez et al., 2009), but our data show that one secondary neuromast will always form between nearby primary neuromasts regardless of their distance (three to five somites apart). Interestingly, the system displays natural variation in the number of primary neuromasts while maintaining the same distribution of organs. The number of mpLL neuromasts in Tg(Eya1:EGFP) ranges from five to eight (Fig. 4D). Larvae with five mpLL neuromasts display secondary organs between a pair of primary organs (100%; n = 70 secondary neuromasts, n = 14 pLLs) and so do larvae displaying eight secondary neuromasts (100%; n = 48 secondary neuromasts, n = 6 pLLs). This observation suggests that the number of secondary neuromasts is not decided by the empty spaces in the surrounding tissues (the occupancy theory), but rather is an intrinsic property of the lateral line systems.

Our previous observations demonstrated that the mpLL neuromasts are formed after vpLL neuromasts, which temporally resembles the formation of new neuromasts from ICs in zebrafish (Ghysen and Dambly-Chaudière, 2007; Grant et al., 2005; López-Schier and Hudspeth, 2005; Núñez et al., 2009; Whitfield, 2005). There, cells that were deposited by the primordium between consecutive neuromasts re-enter the cell cycle in a process that involves Erbb4 signalling and glial cells (Grant et al., 2005; López-Schier and Hudspeth, 2005; Lush and Piotrowski, 2014; Sánchez et al., 2016). We followed secondary neuromast formation in 4D and could not detect proliferating ICs (n = 14 secondary neuromast formation in 11 embryos). Intriguingly, instead we observed that all six to eight ICs group together in the middle of two consecutive primary neuromasts (Fig. 5A,B; Movie 3), start dorsal migration (Movies 2 and 4) and later form a secondary neuromast. In addition, eya1+ cells escape from primary neuromasts and get incorporated in...
Cxcr4b is necessary for secondary organ formation
Secondary neuromasts are generated by individual migration of ICs and cells from primary neuromasts. The Cxcl12a/Cxcr4b/Cxcr7b pathway is crucial for the collective migration of the primordium (David et al., 2002; Haas and Gilmour, 2006; Yasuoka et al., 2004; Valentin et al., 2007) and for individual cell migration of primordial germ cells (PGCs) in medaka and zebrafish (Boldajipour et al., 2008; Doitsidou et al., 2002; Herpin et al., 2008; Knaut et al., 2003; Sasado et al., 2008). Expression of cxcr4b and cxcl12a in medaka (Herpin et al., 2008; Yasuoka et al., 2004) largely matches that reported for zebrafish during primordium migration, with cxc4b expressed in the primordium and in the deposited primary neuromasts, and cxcl12a distributed along the horizontal myoseptum (Fig. S2). At later stages, however, we noticed cxc4b expression in the ICs (Fig. S2), in the migrating cells that form the secondary neuromasts and in the secondary organs (Fig. 6A,B). We complemented this with 4D imaging of Tg(cxcr4b:EGFP) embryos (Fig. S2) to show EGFP+ cells that migrate from primary to secondary organs (Fig. 6C-C‴; Movie 5). By the time secondary neuromasts reached their final position, cxc4b expression was restricted to secondary neuromasts and absent in primary ones (Fig. 6B). The dynamic expression of cxc4b suggests that the same chemokine receptor involved in collective migration of the primordium might play a later role during secondary organ formation.

To functionally test the role of Cxcr4b in secondary neuromast formation, we made use of the cxc4b mutant kazura, which exhibits severe phenotypes in the pLL due to the impaired migration of the primordium (Yasuoka et al., 2004). Although most maternal/zygotic (m/z) kazura mutants do not display pLL neuromasts (59.2%; n=142 pLLs), a proportion manages to generate up to five neuromasts (36.6%; n=142 pLLs) (Yasuoka et al., 2004). In the few cases in which kazura mutants display six or seven neuromasts (4.2%; n=142 pLLs), all were primary organs (Fig. 6D). We only observed neuromasts along the vPLL, meaning they had reached their final position but could not form secondary neuromasts. Our results indicate a role for Cxcr4b in secondary neuromast formation because primary neuromasts in kazura mutants fail to produce secondary organs.

Cxcr7 is necessary for secondary organ formation
Next, we carried out in situ hybridisations for cxcr7, a second receptor for Cxcl12a that in zebrafish is necessary for generating a signalling gradient along the primordium by acting as a sink for Cxcl12a at the rear (Dambly-Chaudière et al., 2007; Donà et al., 2013; Valentin et al., 2007). We detected a clear signal at the rear of the primordium as well as in deposited primary organs (Fig. S2). At later stages, however, cxcr7 is not observed either in the ICs or in forming secondary organs (Fig. 6E,F). The viable mutant yanagi (Sasado et al., 2008; Yasuoka et al., 2004) allows exploration of the function of Cxcr7 during secondary organ formation. Similar to kazura mutants, m/z yanagi mutants displayed a strong phenotype with most pLLs lacking all neuromasts (56.7%; n=60 pLLs) although a fifth of the mutant larvae still display eight or more primary neuromasts (20%; n=60). Among these, however, most larvae showed no secondary neuromasts (66%; n=12 pLLs) (Fig. 6G) and the remaining just one secondary neuromast (33%; n=12 pLLs). Our results indicate that primary neuromasts in yanagi mutants fail to produce secondary organs, and therefore indicate a role for Cxcr7 in secondary neuromast formation.
Directed against chimeric primordium by injecting two guide-RNAs (gRNAs) in the (Haas and Gilmour, 2006). In order to complement our observations located in the middle and rear part eventually form mutant organs are maintained at the front of the primordium whereas mutant cells roles during secondary organ formation. 

On the mutants strongly implies that Cxcr4b and Cxcr7 have distinct picked up by those organs (Movies 8 and 9) (Eya1:EGFP, but are capable of neither coalescing nor being neuromasts, but are incapable of being picked up by those organs (Movies 8 and 9) (n=5). Our 4D data on the mutants strongly implies that Cxcr4b and Cxcr7 have distinct roles during secondary organ formation.

In zebrafish wild-type/cxcr4b chimeric primordia, wild-type cells are maintained at the front of the primordium whereas mutant cells located in the middle and rear part eventually form mutant organs (Haas and Gilmour, 2006). In order to complement our observations in the kazura mutant, we exploited this internal organisation of a chimeric primordium by injecting two guide-RNAs (gRNAs) directed against cxcr4b. Both gRNAs worked in vitro and in vivo on the cxcr4b locus (Fig. S3) and similar approaches have been successfully used in medaka to generate F0 chimeras (Stemmer et al., 2015b). We observed that all injected embryos displayed pLLs on both sides when analysed at the larval stage. A proportion of these, however, displayed an aberrant ratio of primary to secondaries. The autonomous control of the lateral line organs was revealed that most ICs, instead of grouping together in between organs, migrate back to the closest primary organ (Movie 6) (n=8). Secondary organs therefore fail to form in kazura mutants because there are insufficient ICs in place (Movie 7). yanagi mutant ICs, however, are seen dynamically shuttling between primary neuromasts, but are capable of neither coalescing nor being picked up by those organs (Movies 8 and 9) (n=5). Our 4D data on the mutants strongly implies that Cxcr4b and Cxcr7 have distinct roles during secondary organ formation.

Wild-type cells rescue secondary organ formation in kazura and yanagi mutants

To understand how secondary organs fail to form in kazura and yanagi mutants, we followed the dynamics of ICs in both genotypes. Live imaging of Tg(Eya1:EGFP) kazura mutants revealed that most ICs, instead of grouping together in between organs, migrate back to the closest primary organ (Movie 6) (n=8). Secondary organs therefore fail to form in kazura mutants because there are insufficient ICs in place (Movie 7). yanagi mutant ICs, however, are seen dynamically shuttling between primary neuromasts, but are capable of neither coalescing nor being picked up by those organs (Movies 8 and 9) (n=5). Our 4D data on the mutants strongly implies that Cxcr4b and Cxcr7 have distinct roles during secondary organ formation.

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DISCUSSION

Remarkable plasticity in body shape has made teleost fish a very diverse animal class, containing roughly 80% of all vertebrate species. This plasticity comes with inherent problems, i.e. the changing ratio among organ sizes, different proportions of cell types within and expanded regions that need to be innervated and vascularised. We dynamically followed the posterior lateral line formation in an uncharacterised species to reveal a counter-intuitive morphogenetic event, in which a ventral pLL is generated along the myoseptum and migrates ventrally, whereas the midline pLL is generated at ventral positions and migrates dorsally.

Internal organisation of the lateral line system in medaka

We found a variable number of neuromasts in the pLL among medaka larvae. It is unlikely that this reflects polymorphisms in the population, as most larvae display different numbers of neuromasts in the left and right pLLs (73%; n=90 pairs of pLLs). The same genetic information can therefore result in different morphological outputs on each side of the same organism. How is the relative number of midline and ventral pLL neuromasts defined? If the two lines compete for empty spaces along the medaka trunk, we would expect that a fish with a high number of primaries would generate more secondaries, and one with fewer primaries would need several secondaries to occupy all available positions. However, a pair of primary organs generates just one secondary neurast, regardless of the total number deposited by the primordium. Taken together, our data indicate that the decision to generate a secondary organ resides within the lateral system and is not triggered by external cues. The autonomous control of the lateral line organs was highlighted by Ghysen and Dambly-Chaudière in the context of neuromast size (Ghysen and Dambly-Chaudière, 2007), and our results extend that concept to the initial formation of the system. We believe that this mode of lateral line morphogenesis ensures a stereotypic distribution and ratio of mature organs.
Same cells, same molecules, different morphogenetic events

There are three known ways of generating a fish neuromast during homeostatic conditions: (1) direct deposition by the primordium during early embryogenesis (Ghysen and Dambly-Chaudière, 2007; Haas and Gilmour, 2006; Lecaudey et al., 2008; Nechiporuk and Raible, 2008), (2) proliferation of ICs between consecutive neuromasts during the early larval stages (Ghysen and Dambly-Chaudière, 2007; Grant et al., 2005; López-Schier and Hudspeth, 2005; Nuñez et al., 2009; Whitfield, 2005) and (3) neuromast fission or stitching, during late larval and young adult stages (Ghysen and Dambly-Chaudière, 2007; Ledent, 2002; Sapède et al., 2002; Wada et al., 2010). We show that midline pLL neuromasts in medaka are formed by the coalescence of ICs and cell recruitment from primary organs. The proportion of ICs and cells coming from the primary organs varies along the anterior-posterior axis, but includes in all cases the total amount of ICs between primary organs (six to eight Eya1+ cells). Interestingly, this process does not involve cell proliferation, which resumes only when secondary organs reach their final destination (A.S. and L.C., unpublished). We report a novel way to make embryonic neuromasts that results in a new, separate lateral line. Our data using kazura and yanagi mutants indicate that these receptors are being re-utilised by the system to generate more neuromasts, although mediating different processes, i.e. individual cell migration. Overall, eya1-expressing cells use the same chemokine receptors to generate identical neuromasts in two different manners.

Distinct roles for Cxcr4b and Cxcr7 during secondary organ formation

The generation of chimeras wild type/kazura and wild type/yanagi revealed that wild-type cells can rescue both primordium migration and secondary organ formation. The distribution of wild-type EGFP+ cells along the pLL of each mutant suggests similar roles of Cxcr4b and Cxcr7 during primordium migration to those reported in zebrafish. In wild-type kazura chimeras, H2B-EGFP+ cells accumulate towards the most posterior neuromasts (as expected if Cxcr4b is necessary at the leading part of the primordium) whereas in wild-type yanagi chimeras EGFP+ cells accumulate towards the most anterior neuromasts (as expected if Cxcr7 is necessary at the rear of the primordium). The proportion of EGFP+ cells that rescue the mutant phenotype was different in each case. To rescue primordium migration fully in yanagi mutants, the lateral line should contain a majority of wild-type EGFP+ cells; if there are just a few wild-type cells in the...
These will be deposited on the first neuromasts and the primordium will stop caudal migration (observed in two larvae). On the other hand, in kazura mutants, a few EGFP+ cells were enough to rescue full primordium migration. Although we cannot exclude the possibility that putative early defects in primordium migration might contribute to the phenotypes that we report for kazura and yanagi mutants, our results suggest that both Cxcr4b and Cxcr7 are used during secondary organ formation. The high ratio of transplanted cells prevents us from making statements about the specific role of Cxcr7 during secondary organ formation. Integrating the expression data, it might well be that Cxcr7 is operating from the primary organs in an indirect manner. On the other hand, the fact that every secondary organ contains EGFP+ cells indicates that cxcr4b is necessary in the migrating cells initiating the formation of new neuromasts, in accordance with the expression of cxcr4b in ICs. cxcr4b+ ICs could be responding to a Cxcl12a ligand. Based on our data, we cannot state whether cxcr4b+ ICs are migrating in a Cxcl12a-dependent or -independent manner. Interestingly, it has been shown that fibroblast growth factor (FGF) works as an attractant cue for isolated cells towards the primordium during early migration in zebrafish (Breau et al., 2012), and functions as an antagonist for Cxcr4b signalling. Our data suggests that this same interaction could modulate secondary organ formation in medaka, as cxcr4b mutant cells were observed shuttling back to the primary organs instead of coalescing between them. Additional experiments will be needed to address whether a fine-tuning of the opposing roles of FGF and Cxcr4b triggers secondary organ formation in medaka.

**On the diversity of pLL patterns**

The embryonic development of the pLL has been extensively studied in zebrafish, Astyanax mexicanus and Thunnus thynnus (Ghysen et al., 2012, 2010; Pichon and Ghysen, 2004; Sapède et al., 2002), all of which display two pLL primordia during embryonic development and a species-specific pattern of neuromasts at adult stages. This has led to the notion that the plethora of different patterns of pLLs arises primarily during post-embryonic stages of teleost fish, building on largely identical embryonic patterns (Nuñez et al., 2009; Pichon and Ghysen, 2004). Our results complement this assumption by showing pronounced changes during embryonic organogenesis of lateral line patterns and place medaka as the first reported teleost species to have only one pLL primordium building two parallel sensory lines during embryogenesis. This distribution of neuromasts resembles the complete pattern generated by primII in zebrafish (Nuñez et al., 2009). Interestingly, the neuromasts located along the horizontal myoseptum in an adult zebrafish (Ghysen and Dambly-Chaudière, 2007) are lost when primII is ablated during embryogenesis (Nuñez et al., 2009). We believe that this
promordium might constitute a good example of heterochrony between the more basal zebrafish and the neoteleost medaka (Wittbrodt et al., 2002).

Overall, our results show that one primordium generates two different lateral lines in medaka, whereas two primordia are present in zebrafish, Astyanax and tuna. This raises two interesting questions. First, when did medaka lose a primordium during evolution? This can be addressed dynamically by following pL development in close relatives of medaka such as platyfish and stickleback and we believe the Tg(Eya1:EGFP) constitutes an ideal tool. Second, how can a species lose an entire developmental module, i.e. a primordium? Recent work has demonstrated that small changes, either in non-coding sequences or in temporal expression of key regulators, can have drastic effects on the unfolding of morphogenetic processes (Indjeian et al., 2016; O’Brien et al., 2015; Urbansky et al., 2016). The availability of genomic data in different fish species that contain either one or two primordia allows for a comparative analysis, which could yield interesting and novel insights into the evolution of lateral lines, and more generally the evolution of morphogenetic processes.

**MATERIALS AND METHODS**

**Fish stocks**

Medaka (Oryzias latipes) stocks were maintained as previously described (Koster et al., 1997). Fish were kept as closed stocks in a fish facility built according to the local animal welfare standards (Tierschutzgesetz §11, Abs. 1, Nr. 1), and animal experiments were performed in accordance with European Union animal welfare guidelines. The facility is under the supervision of the local representative of the animal welfare agency. Fish were maintained in a constant recirculating system at 28°C on a 14 h light/10 h dark cycle (Tierschutzgesetz 111, Abs. 1, Nr. 1, Haltungserlaubnis AZ35–9185.64 and AZ35–9185.64/BH KIT).

The strains used in this study are: Cab (wild-type population), kazura (cxc4r4b mutant) and yanagi (cxc7 mutant) (Sassano et al., 2008; Yusaoku et al., 2004), Gaudi (SceI vector (Rembold et al., 2006b; Thermes et al., 2002) already containing EGFP, mECFP or H2B-EGFP. For generation of Tg(cxc4r4b:EGFP), a 1.9 kb fragment upstream of the presumptive first exon of the endogenous cxc4r4b locus was amplified from Cod genomic DNA using a specific primer set (forward: ATCCCTGACCACCCACATGA; reverse: AGCCGAGCTCCTCACAAGGC). The PCR fragment was A-tailed and cloned into pGEM T-easy (Promega), and subcloned using Apal/Sall sites into I-cScel vectors (Rembold et al., 2006b; Thermes et al., 2002).

**DIAsp/DAPI staining**

Hair cells in living embryos were detected by using the vital dye 4-Di-2-ASP (Sigma-Aldrich) as previously described (Collazo et al., 1994; Sape et al., 2002). Briefly, live samples were incubated for 5-10 min in a 5 mM DIAsp solution, washed three times in ERM and observed under a fluorescent microscope. Alternatively, samples were treated with DAPI (5 mg/l) for 5 min and washed three times with ERM before mounting them for confocal microscopy.

**Imaging and image analysis**

Embryos were screened and imaged using an Olympus MVX10 binocular coupled to a Leica DFC500 camera, a Macro-scope Nikon AZ100 coupled to a Nikon C1 confocal, and the confocal laser-scanning microscopes Leica TCS SPE (40x oil objective) and Leica TCS SPS II (10x dry, 40x DH20 objectives). For long-term time-lapse imaging, we used a Microscope Slide Temperature Controller (Biotronix). In situ hybridisation pictures were taken using an Axio microscope connected to an HRC camera (Zeiss).

All image analysis was carried out using standard Fiji software. Image stitching was performed automatically using 2D and 3D stitching plug-ins on Imagej or using Photoshop to align images manually. Pseudocolouring of pigments and/or eya1+ spinal cord neurons was achieved using Fiji software.

**Two-photon laser ablation**

The lateral line primordium of Tg(Eya1:GFPs) medaka embryos was ablated either at 2 (early) or at 3 (middle-way) days post-fertilisation (dpf). A TriM Scope 2-photon microscope (LaVision BioTec, Bielefeld, Germany) mounted on a Nikon FN-1 upright stand, equipped with a Chameleon Ultra II femtosecond Ti:Sa laser (Coherent, Dieburg, Germany) and a water-dipping 700 mW at the objective output, depending on the depth of the ablation.

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along with 150 ng/μl of CAS9 mRNA into Tg(Eya1:EGFP) one-cell-stage medaka embryos. The sequences of cxc4r4b gRNAs are GUGAAAACUGGUACUUCCGGAGG and CAAGUGGAUUUCAUACCCGAGG.

**Live-imaging sample preparation**

Dechorionated embryos or hatchlings were anaesthetised using Tricaine (Sigma-Aldrich, A5040-25G). Tricaine (2 g) was dissolved in 500 ml of ddH2O together with 5 g of NaH2P2·2H2O (Grüssing, 12133) and used as a 20× stock solution. Embryos were mounted in 0.6% low melting agarose in ERM (embryo rearing medium) (Rembold et al., 2006a) on glass-bottomed dishes (MatTek corporation). They were covered with 1× Tricaine in 1× ERM to prevent dehydration. After imaging, dechorionated embryos were removed out of agarose and allowed to recover in ERM.

**Whole-mount in situ hybridisation**

Probe generation and in situ hybridisations were performed as previously reported (Stemmer et al., 2015a). Briefly, partial eya1, cxc4r4b, cxc7 and cxc112a cDNA clones were linearised to generate an anti-sense and control sense probes. The fragments were purified with InnuprepPCRpure (Analytic Jena), and used in reactions with T7, SP6 (antisense) or T3 (sense) RNA polymerases and Dig-UTP, to obtain labelled probes that were stored at −20°C in hybridisation solution. Hybridisations were performed overnight at 65°C and samples were incubated afterwards with an antibody against digoxigenin conjugated with AP Fab fragments (1:2000; Roche, 11093274910). Staining was performed using NBT/BCIP (Roche) and anti-digoxigenin conjugated with AP Fab fragments (1:2000; Roche, 11093274910).
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Competing interests
The authors declare no competing or financial interests.

Author contributions
A.S. and L.C. conceived the project and designed the experiments; A.S. performed primordium ablations; A.S. and L.C. analysed the results; A.S., I.K. and L.C. wrote the manuscript.

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References
Amer, A. and Piotrowski, T. (2008). Wnt/P-catenin and Fgf signaling control collective cell migration by restricting chemokine receptor expression. Dev. Cell 15, 749-761.

Boldajipour, B., Mahabaleshwar, H., Kardash, E., Reichman-Fried, M., Blaser, H., Minina, S., Wilson, D., Xu, Q. and Raz, E. (2008). Control of chemokine- guided cell migration by ligand sequestration. Cell 132, 463-473.

Brea, M. A., Wilson, D., Wilkinson, D. G. and Xu, Q. (2012). Chemokine and Fgf signalling act as opposing guidance cues in formation of the lateral line primordium. Development 139, 2246-2253.

Centanin, L., Ander, J.-J., Hoeckendorf, B., Lust, K., Kellner, T., Kraemer, I., Boldajipour, B., Mahabaleshwar, H., Kardash, E., Reichman-Fried, M., Blaser, H., Minina, S., Wilson, D., Xu, Q. and Raz, E. (2008). Control of chemokine-guided cell migration by ligand sequestration. Cell 132, 463-473.

Collazo, A., Fraser, S. E. and Mabea, P. M. (1994). A dual embryonic origin for vertebrate neuroectoderm. Science 264, 426-430.

Coombs, S., Janssen, J. and Webb, J. F. (1988). Diversity of lateral line systems: evolutionary and functional considerations. In Sensory Biology of Aquatic Animals (R. R. Fay and A. N. Popper, eds), pp. 553-593. New York, NY: Springer New York.

Coombs, S., Bleckmann, H., Fay, R. R. and Popper, A. N. (ed.) (2014). The Lateral Line System. New York, NY: Springer New York.

Dambly-Chaudière, C., Cubedo, N. and Ghysen, A. (2007). Control of cell migration in the development of the posterior lateral line. antagonistic interactions between the chemokine receptors CXCR4 and CXCR7/RDC1. BMC Dev. Biol. 7, 23.

David, N. B., Sapédé, D., Saint-Etienne, L., Thissé, C., Thissé, B., Dambly-Chaudière, C., rosa, F. M. and Ghysen, A. (2002). Molecular basis of cell migration in the fish lateral line: role of the chemokine receptor CXCR4 and of its ligand, SDF1. Proc. Natl. Acad. Sci. USA 99, 16297-16302.

Doitsidou, M., Reichman-Fried, M., Stebler, J., Köppner, M., Dörrries, J., Meyer, D., Esquerra, C. V., Leung, T. and Raz, E. (2002). Guidance of primordial germ cell migration by the chemokine SDF-1. Cell 111, 647-659.

Doná, E., Barry, J. D., Valentin, G., Quirin, C., Khmelinskii, A., Kunze, A., Durdu, M., Lahiri, K., Foulkes, N. S. and Wittbrodt, J. (2014). Kremen1 restricts Dllk activity during posterior lateral line development in zebrafish. 141, 3212-3221.

Núñez, V. A., Sarrazin, A. F., Cubedo, N., Allende, M. L., Dambly-Chaudière, C. and Ghysen, A. (2009). Postembryonic development of the posterior lateral line in the zebrafish. Evol. Dev. 11, 391-404.

O’Brien, N. M., Summers, B. R., Jones, F. C., Brady, S. D. and Kingsley, D. M. (2015). A recurrent regulatory change underlying altered expression and Wnt response of the stickleback armor plates gene EDA. Evol. Ecol 105290.

Pichon, F. and Ghysen, A. (2004). Evolution of posterior lateral line development in fish and amphibians. Evol. Dev. 6, 187-193.

Rembold, M., Lahiri, K., Foulkes, N. S. and Wittbrodt, J. (2006a). Transgenesis in fish: efficient selection of transgenic fish by co-injection with a fluorescent reporter construct. Nat. Protoc. 1, 1133-1139.

Rembold, M., Loosli, F., Adams, R. J. and Wittbrodt, J. (2006b). Individual cell migration serves as the driving force for optic vesicle evagination. Science 313, 1130-1134.

Sánchez, M., Ceci, M. L., Gutiérrez, D., Anguita-Salinas, C. and Allende, M. L. (2016). Mechano sensory organ regeneration in zebrafish depends on a population of multipotent progenitor cells kept latent by Schwann cells. BMC Biol. 14, 27.

Sapédé, D., Gompel, N., Dambly-Chaudière, C. and Ghysen, A. (2002). Cell migration in the postembryonic development of the fish lateral line. Development 605-615.

Sasado, T., Yasuoka, A., Abe, K., Mitiy, H., Furutani-Seiki, M., Tanaka, M. and Kondo, H. (2009). Distinct contributions of CXCR4b and CXCR7/RDC1 receptor systems in regulation of PGC migration revealed by medaka mutants kazura and yanagi. Dev. Biol. 320, 328-339.

Stemmer, M., Schuhmacher, L. N., Foulkes, N. S., Bertolucci, C. and Wittbrodt, J. (2015a). Cavefish eye loss in response to an early block in retinal differentiation tool. PLOS ONE 10, e0124633.

Thermes, V., Grabher, C., Ristoratore, F., Bourrat, F., Choulika, A., Wittbrodt, J. and Joly, J.-S. (2002). I-SceI mammalianmediates highly efficient transgenesis in fish. Mech. Dev. 118, 91-98.

Urbanovsky, S., González-Avalos, P., Wosch, M. and Lemke, S. (2016). Folded gastroplasty and T4B drive the evolution of coordinated mesoderm internalization in flies. Elife 5, e13813.
Valentin, G., Haas, P. and Gilmour, D. (2007). The chemokine SDF1a coordinates tissue migration through the spatially restricted activation of Cxcr7 and Cxcr4b. *Curr. Biol.* **17**, 1026-1031.

Venkiteswaran, G., Lewellis, S. W., Wang, J., Reynolds, E., Nicholson, C. and Knaut, H. (2013). Generation and dynamics of an endogenous, self-generated signaling gradient across a migrating tissue. *Cell* **155**, 674-687.

Wada, H., Hamaguchi, S. and Sakaizumi, M. (2008). Development of diverse lateral line patterns on the teleost caudal fin. *Dev. Dyn.* **237**, 2889-2902.

Wada, H., Ghysen, A., Satou, C., Higashijima, S.-i., Kawakami, K., Hamaguchi, S. and Sakaizumi, M. (2010). Dermal morphogenesis controls lateral line patterning during postembryonic development of teleost fish. *Dev. Biol.* **340**, 583-594.

Whitfield, T. T. (2005). Lateral line: precocious phenotypes and planar polarity. *Curr. Biol.* **15**, R67-R70.

Wittbrodt, J., Shima, A. and Schartl, M. (2002). Medaka—a model organism from the far East. *Nat. Rev. Genet.* **3**, 53-64.

Yasuoka, A., Hirose, Y., Yoda, H., Aihara, Y., Suwa, H., Niwa, K., Sasado, T., Morinaga, C., Deguchi, T., Henrich, T. et al. (2004). Mutations affecting the formation of posterior lateral line system in Medaka, Oryzias latipes. *Mech. Dev.* **121**, 729-738.