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In toto imaging of the migrating Zebrafish lateral line primordium at single cell resolution

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ABSTRACT

The zebrafish Posterior Lateral Line primordium (PLLp) has emerged as an important model system for studying many aspects of development, including cell migration, cell type specification and tissue morphogenesis. Despite this, basic aspects of PLLp biology remain incompletely understood. The PLLp is a group of approximately 140 cells which pioneers the formation of the Posterior Lateral Line (LL) system by migrating along the length of the embryo, periodically depositing clusters of epithelial cells, which will go on to form the mature sense organs of the lateral line, called neuromasts. The neuromasts are formed within the migrating PLLp as protoneuromasts: the first protoneuromast is formed close to the trailing end and additional protoneuromasts are formed sequentially, progressively closer to the leading edge of the migrating collective. We imaged the migration of PLL primordia and tracked every cell in the lateral line system over the course of migration. From this data set we unambiguously determined the lineage and fate of every cell deposited by the migrating PLLp. We show that, on average, proliferation across the entire PLLp is weakly patterned, with leading cells tending to divide more slowly than trailing cells. Neuromasts are formed sequentially by local expansion of existing cells along the length of the PLLp, not by self-renewing stem cell-like divisions of a restricted leading population that is highly proliferative. The fate of deposited cells, either within neuromasts or as interneuromast cells (in between deposited neuromasts) is not determined by any obvious stereotyped lineages. Instead, it is determined somewhat stochasitcailly, as a function of a cells distance from the center of a maturing protoneuromast. Together, our data provide a rigorous baseline for the behavior of the PLLp, which can be used to inform further study of this important model system.

1. Introduction

The Posterior Lateral Line is a sensory system in aquatic vertebrates consisting of an array of sensory organs, called neuromasts, distributed along the length of the animal. Each of these organs contains a small bundle of sensory hair cells which detect deflection caused by water flow about the animal (Coombs and van Netten, 2006). These hair cells are innervated by axons of the Lateral Line nerve, which emanate from the Lateral Line ganglion, just caudal to the ear.

The Posterior Lateral Line in zebrafish is established by the Posterior Lateral Line primordium (PLLp), a cluster of approximately 140 cells, which forms posteriorly to the ear and migrates caudally along the horizontal myoseptum toward the tip of the tail. During this migration, the PLLp deposits approximately 5–7 widely spaced neuromasts (NMs), sequentially named L1-L7. At the end of its migration the remaining PLLp cells resolve to form 2–3 closely spaced terminal neuromasts (TNMs) at the caudal end of the tail (reviewed in Chitnis et al. (2012)).

Neuromasts form sequentially as protoneuromasts starting at the trailing end of the PLLp before it begins migration. PLLp migration is initiated after the formation of the first 2–3 protoneuromasts and an additional 4–5 are formed *de novo* within the PLLp during migration at positions progressively closer to the leading edge (Nechiporuk and Raible, 2008). Each protoneuromast contains a central sensory hair cell progenitor embedded within a rosette formed by apical attachment and constriction of surrounding epithelial support cells. The central hair cell progenitor divides, about the time the protoneuromast is deposited, to form a pair of sensory hair cells that will differentiate to have opposing responsiveness to the direction of water flow (Lopez-Schier et al., 2004). Surrounding cells, not specified as hair cell progenitors,

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remain as support cells. These cells provide structural support to the hair cells and serve as a pool from which additional hair cell progenitors are sequentially specified during growth and regeneration of the deposited neuromasts (reviewed in Ma and Raible (2009)). Cells in the PLLp that are not epithelialized and incorporated into proto-neuromasts, are continuously deposited as so-called interneuromast cells in between the deposited neuromasts (Grant et al., 2005; Lopez-Schier and Hudspeth, 2005; Lush and Piotrowski, 2014).

The formation of protoneuromasts is coordinated by the interaction of the Wnt and Fgf signaling pathways. Wnt signaling, which dominates in a leading domain, drives expression of two Fgf ligands, Fgf10a and Fgf3. Simultaneously, Wnt activity drives expression of two inhibitors of Fgf receptor signaling, Dusp6 (Matsuda et al., 2013) and Sef (Aman and Piotrowski, 2008). As a result, cells that are the source of FGF ligands cannot themselves respond to this signal. Instead the Fgfs activate Fgf receptors in a trailing domain, outside the inhibitory influence of Wnt signaling. Activation of the Fgf receptor initiates expression of a diffusible inhibitor of Wnt signaling, Dkk1b, which facilitates establishment of stable Fgf signaling centers (Aman and Piotrowski, 2008). Once established, activation of Fgf signaling coordinates formation of protoneuromasts; it promotes the formation of epithelial rosettes (Lecaudey et al., 2008; Nechiporuk and Raible, 2008) and simultaneously drives expression of the transcription factor Atoh1a (Lecaudey et al., 2008; Nechiporuk and Raible, 2008), which gives protoneuromast cells the potential to become sensory hair cells. Fgf signaling and Atoh1a drive expression of Notch ligands DeltaA and DeltaD, respectively. The DeltaA/DeltaD expressing cells activate Notch in their neighboring cells, which, in turn, inhibits expression of *atoh1a* in these cells (Itoh and Chitnis, 2001; Matsuda and Chitnis, 2010). This process of "lateral inhibition" mediated by Notch signaling restricts *atoh1a* expression to a central cell in the protoneuromast, which is specified as a hair cell progenitor.

While the signaling mechanisms underlying the formation of protoneuromasts are broadly accepted, many questions remain about the specific mechanisms that time both formation and deposition of protoneuromasts and determine the fate of cells in the PLLp. Early lineage studies suggested that a small number of leading-edge cells are part of a progenitor zone that contributes to formation of new neuromasts once already-formed proto-neuromasts are deposited (Nechiporuk and Raible, 2008). Subsequent work identified leading cells (approximately the leading two thirds of the primordium) as a proliferative population based on high levels of BrdU incorporation (Aman et al., 2011). This high level of BrdU incorporation was thought, at least in part, to be dependent on Wnt signaling, which determines expression of one of its effectors, Lef1, in the leading one third of the primordium (Aman and Piotrowski, 2008). Numerous studies have suggested roles for Wnt signaling in specification, maintenance and self-renewal of progenitors (Grigoryan et al., 2008; Kalani et al., 2008). Together, these observations could be interpreted to suggest that a Wnt-dependent self-renewing progenitor population proliferates to seed a field of trailing cells that contributes to formation of sequentially deposited neuromast and interneuromast cells, and that exposure to Fgf signaling promotes incorporation of these cells into epithelial protoneuromasts. However, it remained unclear if indeed a restricted set of leading cells serves as a proliferative self-renewing progenitor population or whether instead the entire length of the primordium represents a population of undifferentiated progenitors that sequentially reorganizes to form protoneuromast starting at the trailing end, leaving undifferentiated "progenitors" in a progressively smaller leading domain of the migrating primordium.

Though there is a broad framework for understanding how cell fate, morphogenesis and collective migration of the PLLp are coordinated, many basic aspects of the biology of the PLLp, including proliferation patterns, lineage relationships and the dynamics of neuromast formation were incompletely characterized. We reasoned that *in toto* imaging of the migrating Lateral Line primordium would unambiguously resolve such questions. Additionally, a detailed understanding of lateral line development at the single cell level will greatly contribute to our ability to analyze how mutations affect cell behaviors. To this end, we conducted time-lapse imaging of the PLLp, first at low resolution for a number of embryos, and then again for three independent embryos at a resolution which allowed us to track and catalog every single cell in the PLLp from early in migration until termination (approximately 24 h). From this dataset, we constructed "digital PLLps" in which we could query the entire cell lineage throughout migration. We find that leading cells do not form a separate domain with a self-renewing population of progenitors that contributes to sequential formation of protoneuromasts in the trailing zone. Instead, protoneuromasts are formed by local expansion of existing cells along the length of the PLLp, under the influence of FGF signaling centers that form sequentially in the wake of a progressively shrinking leading Wnt system. In the context of this process, a small population of about a dozen cells, located at the leading end of the primordium at the beginning of migration, eventually expands to contribute almost exclusively to the terminal neuromasts by the end of migration, and not to deposited neuromasts.

2. Material and methods

2.1. Zebrafish care

Adult Zebrafish (*Danio rerio*) were maintained under standard conditions and stages according to Kimmel (Kimmel et al., 1995). All embryos were generated by natural spawning and maintained at 28.5 °C.

2.2. Low resolution time-lapse analyses

To track larger numbers of embryos over time, we first embedded *Tg(clbnb:lynGFP)* embryos (Haas and Gilmour, 2006) in 0.75% low-melt agarose and imaged fish at 5-min intervals using a 20×0.75NA air-immersion objective lens on a Leica SP5 confocal microscope. From these movies we manually annotated all dividing cells and the position of each neuromast (judged by the central accumulation of lyn-GFP) and the leading edge. Neuromast deposition was classified as the time at which the trailing neuromast was separated from the main PLLp by an interneuromast population two cells in width.

2.3. High resolution tracking and classification

For high resolution tracking, embryos of either *Tg(clbnb:lynGFP)* or *Tg(clbnb:lynGFP)* outcrossed to *Xa231:Gal4; UAS:mTagRFP* were mounted in 0.75% low-melt agarose and imaged at two minute intervals using a 40×1.2NA water-immersion objective lense on a Leica SP5 confocal microscope with ~1 μm z-resolution starting at approximately 22hpf until termination of migration. Images were aligned and stitched together with a moving reference frame centered around the migrating PLLp. Custom scripts in ImageJ were used to automatically extract either membrane outlines or nuclei after manual seeding of each position at every time point. Each completed cell track was then manually validated to ensure complete accuracy. Tracked objects were maintained both as ROIs and as arrays of centroid positions. Fates were assigned in the last frame a cell was visible into one of the following groups: Neuromast (NM), Interneuromast (IN), Terminal Neuromast (TNM) or Different Fate (DF). DF refers to cases in which a cell divides and both daughter cells could not themselves be classified into a single compartment. In the cases where both daughters contributed exclusively to the same compartment, the parental cell was also classified as contributing to that compartment. To classify cells at the margin of neuromasts to a neuromast or interneuromast fate, cells with visible connections to the neuromast center were classified as NM; those without were classified at IN.