

Reviewed Preprint

v1 • May 29, 2024

Not revised

Reviewed Preprint

v2 • March 31, 2026

Revised by authors

✉ For correspondence:

fx220@cam.ac.uk

megason@hms.harvard.edu

* Equal contribution

Competing interests: No competing interests declared

Funding: See [page 16](#)

Reviewing editor: Giulia Boezio, The Francis Crick Institute, United Kingdom

© 2024, Xiong et al. This article is distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use and redistribution provided that the original author and source are credited.

Heterogeneity of Sonic Hedgehog response dynamics and fate specification in single neural progenitors

Fengzhu Xiong^{1,2,*} ✉, Andrea R Tentner^{1,*}, Sandy Nandagopal¹, Tom W Hiscock^{1,3}, Peng Huang⁴, Sean G Tsung-Megason¹ ✉

¹Department of Systems Biology, Harvard Medical School, Boston, United States • ²Gurdon Institute and Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, United Kingdom • ³Institute of Medical Sciences, School of Medicine, Medical Sciences & Nutrition, University of Aberdeen, Aberdeen, United Kingdom • ⁴Department of Biochemistry & Molecular Biology, University of Calgary, Calgary, Canada

eLife Assessment

This study presents an **important** study of the relationship between morphogen signaling and cell fate choices in the forming zebrafish neural tube, addressing a topical question in developmental biology. The authors provide a **solid** characterization of the precision limit for gene regulatory networks interpreting Shh, with single-cell resolution and state-of-the-art in vivo approaches. While the depth of analysis is restricted, particularly by the number of cell traces, the study will be of interest to developmental biologists interested in cellular decision-making.

<https://doi.org/10.7554/eLife.96980.2.sa3>

Abstract

During neural tube patterning, a gradient of Sonic hedgehog (Shh) signaling specifies ventral progenitor fates. The cellular response to Shh is processed through a genetic regulatory network (GRN) to specify distinct fate decisions. This process integrates Shh response level, duration and other inputs and is affected by noise in signaling and cell position. How reliably Shh response profile predicts the fate choice of a single cell is unclear. Here we use live imaging to track neural progenitors in developing zebrafish and quantify both Shh and fate reporters in single cells over time. We found that there is significant heterogeneity between Shh response and fate choice in single cells. We quantitatively modeled reporter intensities to obtain single cell response levels over time and systematically evaluated their correlation with cell fate specification. Motor neuron progenitors (pMN) exhibit a high degree of variability in their Shh responses, which is particularly prominent in the posterior neural tube where the Shh response dynamics are similar to those of the more ventrally fated lateral floor plate cells (LFPs). Our results highlight the precision limit of morphogen-interpretation GRNs in small and dynamic target cell fields.

Introduction

Morphogens pattern tissue by acting over space in a concentration dependent manner (Wolpert 1969 [✉](#); Kicheva and Briscoe 2023 [✉](#)). Biochemically, morphogen molecules may either regulate transcription directly (Driever and Nusslein-Volhard, 1989 [✉](#)), or through a specific transducer following a cascade of intracellular interactions - a signaling pathway. The latter involves additional, potentially non-linear steps of signaling. A prominent example of morphogen patterning is the vertebrate ventral neural tube where the morphogen Sonic hedgehog (Shh), produced from the notochord and floor plate, forms an extracellular gradient. Through its

transcriptional effector Gli, this Shh gradient specifies different neural progenitor domains along the ventral-dorsal axis (Jessell 2000 [↗](#), Figure 1A [↗](#)). In this system, Gli activity profiles (cellular responses) regulate the expression of multiple homeobox transcription factors (fate genes) (Briscoe et al., 2000 [↗](#)). These fate genes regulate their own and each other's expression as a genetic regulatory network (GRN); this network finally settles into one of several discrete and stable states that correspond to different neuronal fates (Lek et al., 2010 [↗](#); Balaskas et al., 2012 [↗](#)). On the basis of population-level observations of the Shh pathway and transcriptional network, different aspects of the Shh signaling/Gli activity profile have been proposed to control cell fate choices: e.g., threshold levels (presumably by different affinities between Gli and fate gene enhancers, Ericson et al., 1997 [↗](#); Oosterveen et al., 2012 [↗](#)), duration of response (sustained Gli levels under the adaptive negative feedback of the Patched [Ptc] receptor, Dessaud et al., 2007 [↗](#)) and the time-integrated response level (Dessaud et al., 2010 [↗](#)). As these response dynamics are integrated through the GRN towards fate decisions, inherent noise in gene expression (Rosenfeld et al., 2005 [↗](#)) may generate heterogeneity in the response-fate correlation, despite the ability of the GRN to increase robustness (Balaskas et al., 2012 [↗](#)). In addition, our previous work reveals extensive cell movement (positional noise) and a role of cell sorting in refining the boundaries of fate domains in the zebrafish neural tube after cells are specified (Xiong et al., 2013 [↗](#)), which are mediated by an adhesion code downstream of cell fate choices (Tsai et al., 2020 [↗](#)). These studies suggest a possible limit of precision in morphogen signal interpretation in fate specification. To test this possibility, single cell observations using live imaging of transgenic reporters to simultaneously monitor both morphogen response and cell fate choices *in vivo* are required (Xiong and Megason, 2015 [↗](#)). The zebrafish neural tube, with established reporter lines of Shh signaling and neural progenitor fates (Huang et al., 2012 [↗](#); Xiong et al., 2013 [↗](#); Tsai et al., 2020 [↗](#)), provides a feasible model system to perform this analysis.

Results

Live tracking of individual neural progenitor fate choices in zebrafish embryos

To enable a direct, single-cell quantification of Shh response dynamics and fate choice, we developed an imaging protocol using double transgenic reporters along with a nuclear or membrane localized EBFP2 for cell tracking (Figure 1B [↗](#)). We combined the *tgBAC(ptch2:kaede)* reporter whose Kaede expression reports Shh response (Huang et al., 2012 [↗](#)) and a second transgenic reporter (e.g., *tg(nkx2.2a:mgfp)* [Ng et al., 2005 [↗](#)], *tg(olig2:gfp)* [Shin et al., 2003 [↗](#)], Figure 1C [↗](#)) whose expression levels indicate different fate choices (Xiong et al., 2013 [↗](#)). Movies with good spatial-temporal coverage of the neural plate/tube (e.g., Movie S1 [↗](#)) were analyzed by single cell tracking in GoFigure2, an open-source software we previously developed for image analysis (Xiong et al., 2013 [↗](#)). By manually placing a seed at the center of the same cell at each time-point in the dataset, we generated a series of spherical segmentations inside the cell, which capture the fluorescence intensity of each reporter and the position of the tracked cell over time. Together, this time-dependent positional and reporter intensity information represents what we call a 'neural progenitor track' (Figures 1D-E [↗](#), Movie S2 [↗](#)). We analyzed over 200 randomly chosen tracked cells to cover the ventral progenitor types in the zebrafish neural tube: the medial floor plate cells (MFPs), lateral floor plate cells (LFPs), motor neuron progenitors (pMNs) and "More Dorsal" cells (e.g., p2,p1,p0 progenitors) (Figure 1A [↗](#); Xiong et al., 2013 [↗](#)). These cell fates form distinct domains highly conserved among vertebrates and prescribe the pattern of neurogenesis (Jessell 2000 [↗](#); Lewis and Eisen, 2003 [↗](#)). In zebrafish, MFPs along with the notochord are the source cells of Shh; LFPs, pMNs and More Dorsal cells depend on Shh signaling for correct specification (Park et al., 2004 [↗](#); Huang et al., 2012 [↗](#)). Taking advantage of the reporter expression and the stereotypic position at the end time point of each track, we identified and assigned different fates to the tracked neural progenitors (Xiong et al., 2013 [↗](#)). We imaged cells from both the anterior spinal cord (approximately somite levels 4-9) as well as the posterior

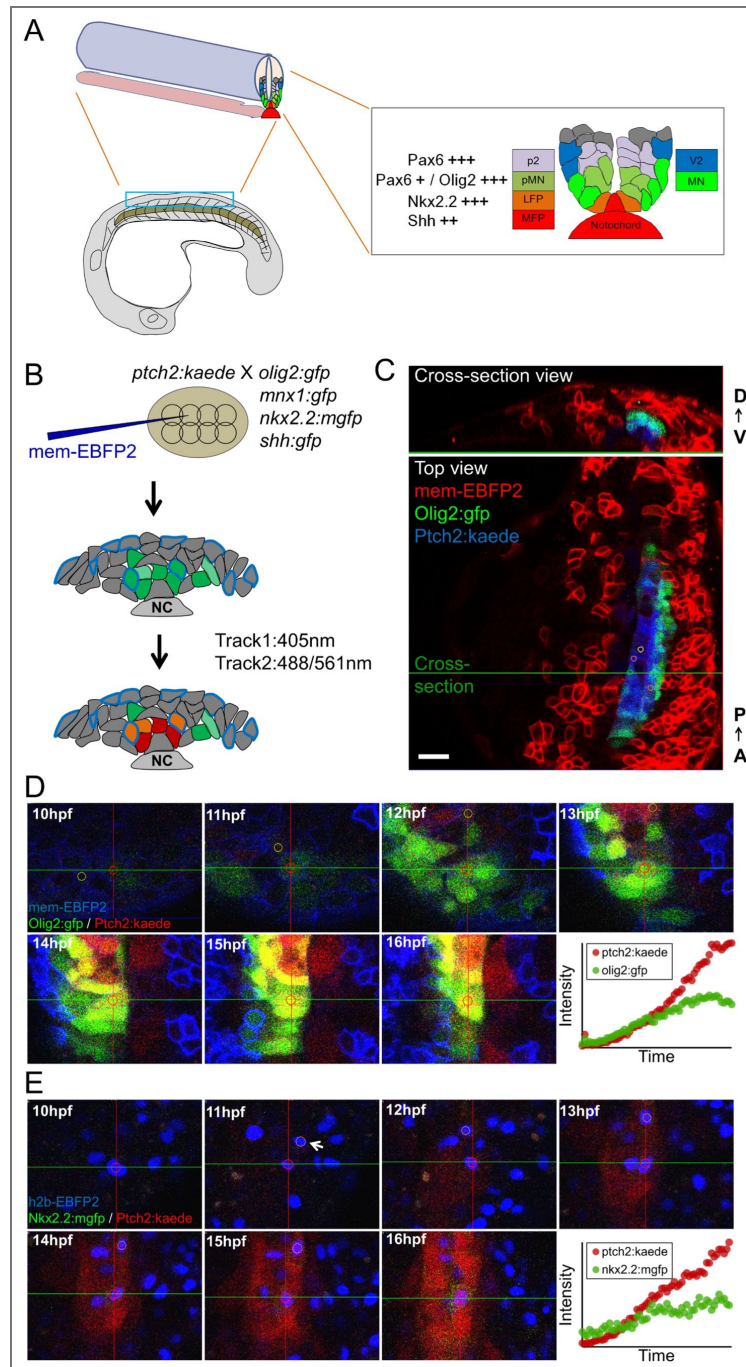


Figure 1. Imaging zebrafish neural tube development and single cell tracks of different fates.

(A) Schematic of ventral neural tube anatomy and pattern in zebrafish (partially adapted from Xiong et al., 2013). MFP, medial floor plate; LFP, lateral floor plate; pMN, motor neuron progenitors (MN, motor neurons); p2, V2 interneuron progenitors (V2, V2 interneurons). (B) Labeling (at 8-16 cell stage) and imaging (at neural plate, neural keel and neural tube stages) scheme for acquiring three channels at the same time. The 405 laser in the first scan line excites the BFP cell tracking marker as well as converting Kaede(Green) to Kaede(Red). NC, notochord. (C) Example data. Small circles on the image show manual segmentations generated on cells using GoFigure2 software. D-V, dorsal-ventral; A-P, anterior-posterior. Scale bar: 20µm. (D) A PMN track in a *mem-ebfp2, ptch2:kaede/olig2:gfp* dataset. Circles in the cells show a 2D view of the spherical segmentations generated using GoFigure2 software. (E) A LFP track in a *h2b-ebfp2, ptch2:kaede/nkx2.2:mgfp* dataset. Arrow indicates a sister cell of the centered track. Note that while this transgenic reporter has a membrane tag, the spherical segmentation is still capable of measuring the much weaker cytoplasmic fluorescent signal. See also Movie S1.

spinal cord (approximately somite levels 9-14). The shape, size and morphogenetic movements of both the Shh sending and receiving tissues vary along the AP axis (Xiong et al., 2013 [DOI](#)), thus allowing us to sample a diversity of input-output (i.e., Shh response-fate choice) relationships.

Heterogeneity is pervasive in Shh response dynamics and fate choice in single cells

Our set of ~200 single cell tracks provide a first glimpse into the dynamics of Shh interpretation *in vivo*. The main new insights come from being able to observe the dynamics of two nodes of the GRN in the same live cell, namely a Shh response reporter (*ptch2:kaede*) and a fate reporter. To compare our data with previous studies, we first analyzed the average Shh response behavior of cells within each fate group (calculated by simple averaging of single cell tracks within each of the different fates). In both the anterior and posterior spinal cord in zebrafish (Movie S3 [DOI](#) and S4 [DOI](#), respectively), the average LFP shows the fastest increase of Kaede intensity, while the average pMN shows intermediate increase and the average More Dorsal cell is the slowest (Figures 2A,C [DOI](#)). Accordingly, the *olig2:gfp* reporter is highly expressed in the average pMN but comes to a plateau after initial expression in the average LFP (Figures 2B,D [DOI](#)). These data are consistent with observations that these cell types require different levels of Shh for specification (Ericson et al., 1997 [DOI](#); Park et al., 2004 [DOI](#)) and that in amniotes the p3 progenitors (equivalent to LFPs in fish) initially express the pMN marker *olig2* but then shift to express *nkx2.2* with continued and/or increasing Shh exposure (Jeong and McMahon, 2005 [DOI](#); Dessaud et al., 2007 [DOI](#)).

We next ask whether single cells behave stereotypically like the averaged cell. Strikingly, at the single cell level, we observed high variability in Shh-signaling reporter dynamics across cells of the same fate and some overlap of cells that finally take on different fates (Figure 2A'-D' [DOI](#)). There are examples of cells that have similar *ptch2:kaede* traces but distinct fate reporter traces (e.g., in Figure 2E [DOI](#), track 1 vs. 2, 3 vs. 4), as well as cells with very different *ptch2:kaede* traces but similar fate reporter traces (e.g., in Figure 2E [DOI](#), track 1 vs. 3). These data show that single cells have heterogeneity in the correlation between Shh response and fate choice. Cells that choose the same fate could have distinct Shh responses and cells that have very similar Shh responses may make different fate choices.

Quantifying reporter activity dynamics from fluorescent intensity tracks

Given the apparent heterogeneity and overlap in the single cell track data, are there certain features in the reporter dynamics that predict fate outcome more accurately? In other words, are the apparently very similar *ptch2:kaede* tracks (as seen in Figure 2E [DOI](#)) different in a certain way that could explain their distinct fate choices? Our tracks offer us an opportunity to address this question by quantifying and systematically classifying Shh signaling activity, namely the transcription rate of the *ptch2* reporter, from the fluorescence intensities. However, the intensity measurements must be handled carefully and interpreted with consideration of experimental limitations to provide useful insights.

First, while the fluorescence intensity levels measured in single cells by image segmentation are in principle proportional to the reporter protein concentration (Figure 3A [DOI](#)), the acquisition and analysis processes may introduce several errors: bleaching, bleed-through, saturation, and segmentation measurement error. Our sampling frequency is even lower here than our previous work, in which other imaging settings were similar and quantification of fluorescence signals indicated that bleaching was not significant (Xiong et al., 2013 [DOI](#)), suggesting that the bleaching factor can be ignored here. To account for bleed-through, which refers to the phenomenon of signal from one fluorophore contributing to another during simultaneous acquisition, we applied a percentage subtraction from each channel after determining the bleed-through ratio using all data points (methods). To account for saturation, which refers to signal intensity exceeding the dynamic range of detection causing an artificial signal plateau, we removed data points where the fraction of saturated pixels exceeds 5% within segmented cells (methods). To handle noise that

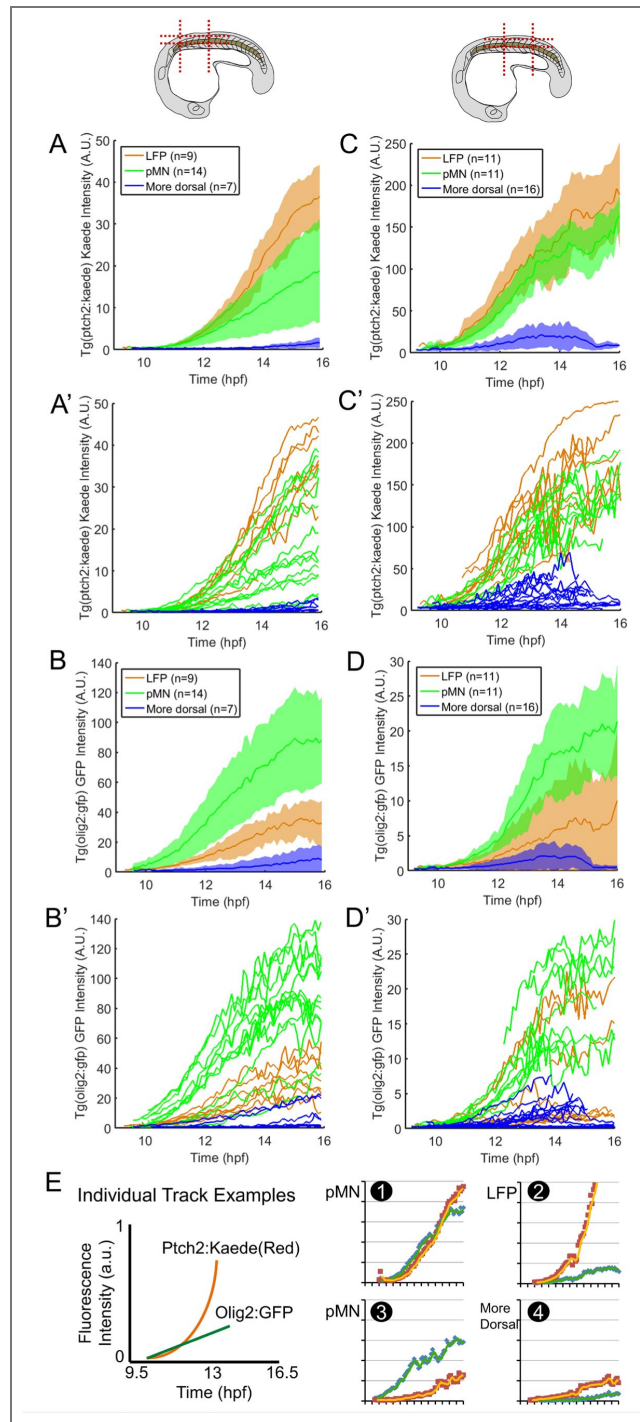


Figure 2. Dynamics and heterogeneity of Shh response and fate reporters in average cells and single cells.

(A-D) Average and single (A'-D') tracks from a *ptch2:kaede/olig2:gfp* movie focusing on the anterior spinal cord (indicated by the schematic, A-B), and the posterior spinal cord (C-D), respectively. Colored shades around the average tracks represent $\pm$ S.D. Individual *ptch2:kaede* traces (A',C') show significant overlap between fates. Note that intensity units are not comparable between different datasets (e.g., between A and C) due to variation in expression, sample depth, mounting position, and imaging settings. The average tracks appear noisier before 10hpf and after 15hpf due to some single cell tracks not being long enough temporally (e.g., a tracked cell leaves the field of view) therefore fewer data points are available for averaging at the ends. LFP, Lateral floor plate cells; pMN, motor neuron progenitors. See also Movies S3, 4. (E) Example single tracks that show very similar *ptch2:kaede* response dynamics but very different *olig2:gfp* expression, and vice versa. The schematic shows the axis ranges and labels, and legends for the two reporter traces.

arises from random fluctuations of segmentation measurement between time points (technical errors), we applied a moving-average smoothing to the tracks over windows of 6 time points (36min, see Figure 6C for effects of varying this time window). This operation causes some short-time scale features of the tracks (which are otherwise not possible to analyze due to technical errors in the measurements) to be lost, but it preserves trends on longer time intervals (Figure 3B). We further confirmed the mitigation of the impact of technical errors by re-tracking the same cells, where the average variation of intensity measurement over 6 time points is consistently <5% (data not shown). Together this pipeline transforms raw tracks to an intensity track that reasonably approximates the reporter protein concentration dynamics in single cells. Note that other factors (e.g., cell growth, elongated cell shapes) may still affect the fluorescence intensity measurement independently of Shh signaling activity) and not accounted for with these datasets that do not contain a cell membrane label.

Second, in order to estimate the cellular Shh response/Gli activity dynamics (reflected by the transcription dynamics at the *ptch2:kaede* reporter), we investigated the relationship between fluorescent intensity (I) and the transcription rate of *kaede* (x) (Elowitz and Leibler, 2000). Since Kaede and GFP proteins can be considered as stable on the time-scale of our experiments (Ando et al., 2002; data not shown), assuming a constant coefficient (c_1) for the rate of Kaede translation and maturation as a function of *kaede* mRNA level (m), the intensity change rate of Kaede (Figure 3C) is given by:

$$\frac{dI}{dt} = c_1 m \quad (1)$$

m is in turn a function of transcription and mRNA stability, given in the form of half-life ($t_{1/2}$):

$$\frac{dm}{dt} = x - m \frac{\ln 2}{t_{1/2}} \quad (2)$$

Combining (1) and (2) we have a simplified relationship between measured fluorescence intensity and Shh response (represented by x):

$$cx = \frac{d^2 I}{dt^2} + \frac{dI}{dt} \cdot \frac{\ln 2}{t_{1/2}} \quad (3)$$

c is a constant. Note that the fluorescent signal is time delayed due to lags caused by transcription, translation, and maturation of the fluorescent protein, therefore our raw tracks do not show reporter responses (transcription) in real time. Importantly, use of destabilized RNA or proteins would not reduce this lag, but only diminish reporter intensity thus making their measurement noisier. Furthermore, because of the time and detection sensitivity differences between Kaede and GFP, we are prevented from cross-correlating the two reporter channels on the same time axis. Despite these limitations, equation (3) still allows us to estimate the Shh response dynamics in each single cell and compare that between different cell fates within each dataset.

To validate equation (3) and estimate reporter mRNA half-life ($t_{1/2}$) *in vivo*, we first applied a heat-shock pulse on transgenic embryos with a *hsp70l:EGFP* transgene (Figures 4A; Halloran et al., 2000), constructed with an SV40 polyadenylation sequence like the *ptch2:kaede* transgene). This causes a defined pulse of transcriptional output at the reporter (Rieger et al., 2005). According to equation (3), the GFP intensity change that follows should reflect the exponential decay of the *gfp* mRNA. Indeed, single cell tracks we generated provide an excellent fit with the model's prediction (Figure 4B), allowing us to estimate the mRNA half-life to be around 1.7 hours. Next, we performed *in situ* hybridization of *kaede* after sequential chemical inhibition of Shh response using cyclopamine (thereby stopping new *kaede* mRNA synthesis) and compared the overall mRNA signal with controls for signal level over a series of time points (Figure 4C; Huang et al., 2012). This method suggests a ~3-hour mRNA half-life. The two estimations are thus congruent on an *in vivo* half-life of fluorescent reporter mRNAs somewhere between 1-4 hours (effects of varying this parameter are assessed in Figure 7B). These data support the validity of equation (3) and enable us to use it and the estimated half-life to infer the dynamics of the *ptch2* transcription rate (i.e. Shh signaling response) in our single neural progenitor tracks (Figure

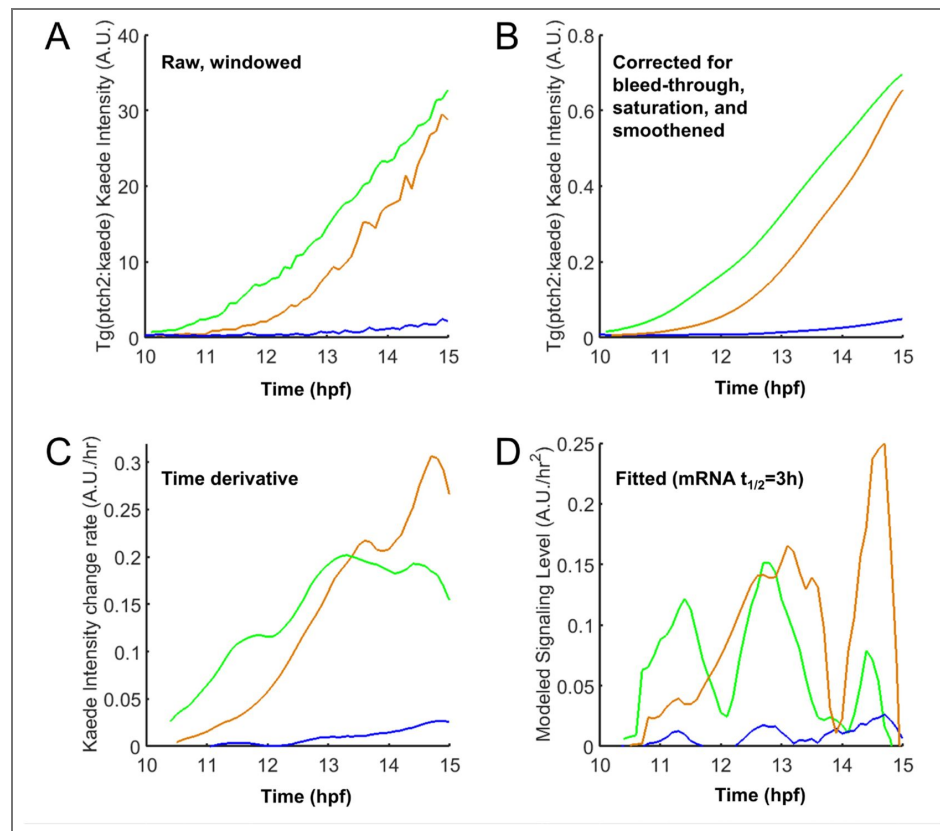


Figure 3. Estimation of dynamic reporter activity from fluorescence intensity measurements

(A) Example raw tracks (a pMN [green], a LFP [brown] and a More Dorsal cell [blue] each). The tracks were trimmed to contain only data points between 10 and 15 hpf as this time window is best covered in the tracks. Before 10 hpf the signal is low and by 15 hpf most progenitors have become specified (Xiong et al., 2013). (B) Tracks in (A) after multiple filter corrections as described in the text. Main trends over long time windows (>0.5 hours) are preserved while short fluctuations are removed by a moving-average smoothing operation. (C) Rate of change in intensity by the first time derivative of (B). (D) Modeled signaling level (*ptch2:kaede* transcription rate) of the same tracks using equation (3). Note the different peak height and duration of different cells. Varying the half-life value between 1 to 5 hours does not substantially change these results (data not shown).

3D [3D](#)). Note that as [equation \(3\)](#) contains second derivative with respect to time of the intensity, this fitting is prone to measurement noise and requires good time resolution. However, due to our smoothing operation, sharp and short-time response changes have been filtered out. Our subsequent comparisons only consider those features that span over at least 0.5-hour windows (e.g., the peaks and valleys seen in [Figure 3D](#)) which are comprised of at least 5 time-point measurements and describe trends that can readily be seen in the raw intensity track ([Figure 3B](#)). This compromise minimizes the impact of technical errors in the analysis at the expense of model resolution.

Systematic analysis of Shh response heterogeneity and association with fate outcome

The processed tracks allow us to gain deeper insights into the characteristics and sources of heterogeneity. First we performed K-means clustering of *ptch2:kaede* dynamics in all cells ([Figure 5A](#)). The resultant clusters are then compared in terms of their contribution to the LFP, pMN and more Dorsal fates ([Figure 5B](#)). We found that large clusters all show fate heterogeneity between “adjacent” fates (LFP and pMN, pMN and more Dorsal), such as Clusters 1,2,3,5 and 7. In contrast, a given cluster does not contribute both to LFP and more Dorsal, with the only exception in Cluster 11. These data show that the heterogeneity between Shh responses and fate choices is pervasive across distinct types (i.e. clusters) of response profiles, and primarily presents itself around progenitor domain boundaries. Over a larger spatial scale (such as between LFP and more Dorsal), the response-fate relationship can be considered predictive and accurate at single cell level.

To explore the sources of signal heterogeneity, we compared the average and variation of Shh response by fates in different datasets ([Figure 5C](#)). The pMN tracks showed the most variability in all datasets while LFP and more Dorsal tracks are more similar on average and less variable. A striking feature that stands out is the similarity between pMN and LFP tracks in the posterior dataset, where the average pMN response appears as strong as the LFPs. To account for the heterogeneity in timing of response initiation, we used model-fit response levels ([Figure 3D](#)) to identify the response initiation time and re-aligned the tracks by this time point ([Figure 5D](#)). The re-aligned pMN average tracks are more similar to each other in the anterior datasets and show reduced variability, suggesting that some of the variability can be attributed to differences in timing of reporter activation. The posterior pMN tracks remain distinctly heterogeneous, suggesting a major contribution of variability is associated with the anterior-posterior level of the neural tube, which carries differences in tissue size and shape, cell movement, and morphogen source, among others.

Correlation of key aspects of Shh response with fate outcome

To further compare the correlation of some key dynamic features of morphogen response to cell fate outcome between the anterior and posterior neural tube, we used the model-fit responses to calculate the maximum transient response level (the peak Shh response within the track), the average response level (average Shh response over 10-15hpf), and the response time (the amount of time with an above-basal Shh response, which may include multiple durations of response) in the tracked time windows. To give a visual indication of how well each metric correlates with the graded fate outcome, we ranked single cell tracks by the value of each metric high-to-low to make a vertical “French flag”: the sharper the flag, the stronger the correlation. The sharpness is quantified by the percentage of correct fate prediction by the metric thresholds. A value near 50% (coin toss) would suggest the metric does not correlate with fate choice at all, whereas 100% would suggest the metric makes perfect prediction. For example, when we use two late-stage cell position metrics, the lateral-medial/dorsal-ventral (LMDV, this distance refers to the axis along which cells are being patterned which is initially lateral-medial because patterning begins at the neural plate which then gradually transitions to dorsal-ventral as the neural tube is formed) should do well in the flag correlation while the anterior-posterior (AP) position should do poorly since we are trying to predict fates across the DV axis ([Figures 6A](#)). Indeed, we find that the LMDV position is highly

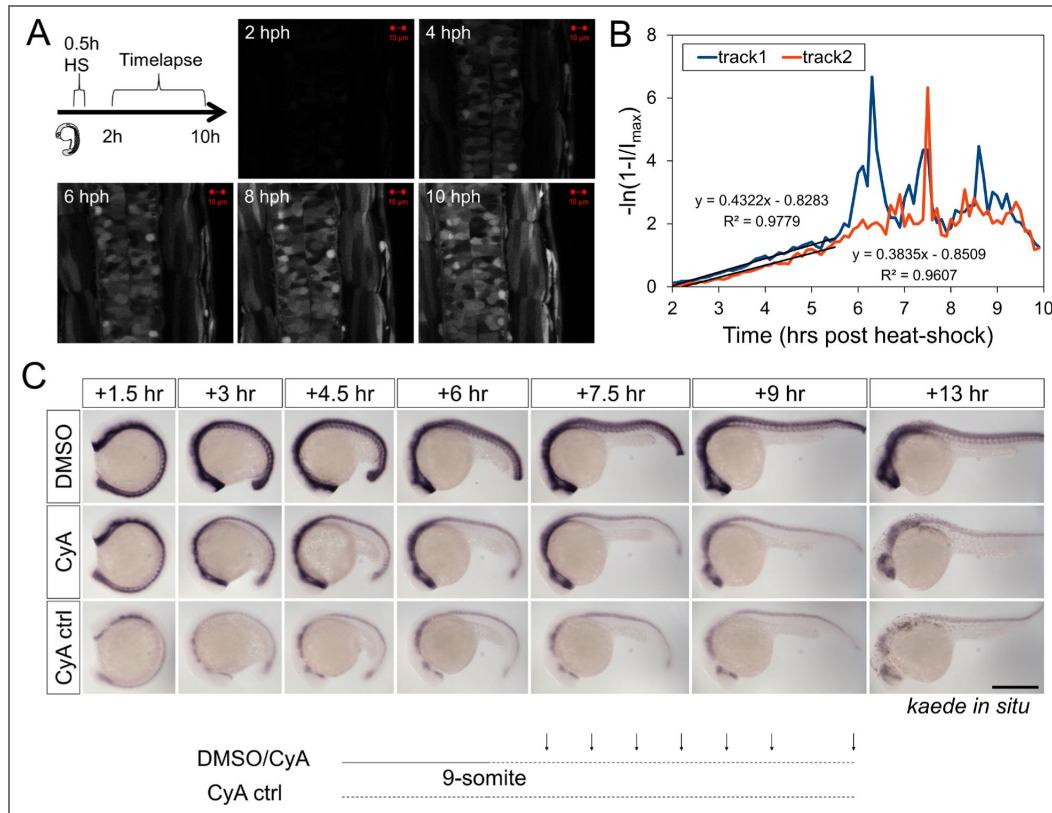


Figure 4. Estimation of fluorescent reporter mRNA half-life.

(A) Heat-shock pulse induction of GFP and timelapse imaging (schematic). 24hpf *tg(hsp70l:EGFP)* embryos were shocked for 30 minutes in pre-warmed 37°C egg water in a heat block. Imaging began 2 hrs post heat-shock (hph, when the fluorescence was first detectable). (B) Cell tracks of *hsp70l:EGFP* to validate equation (3) and estimate mRNA stability. Assuming that the heat-shock between $t=0$ and $t=0.5$ causes a transient pulse of mRNA production, the intensity track then covers the simple decay period of mRNA (as no more mRNA is produced after the pulse). The decay is exponential according to equation (3) and governed by the mRNA half-life. The fluorescent intensity increase rate then should correspondingly decrease exponentially towards a plateau value, predicting a linear relationship between time and $-\ln(1-I/I_{max})$, in which I is raw intensity and I_{max} is the plateau value of I in later times (when no more mRNA is left and new fluorescent protein production stops). This predicted linear relationship is found for the tracks suggesting the simple model correctly describes the process. The slope of the lines (~ 0.4) suggests the mRNA half-life of *hsp70l:egfp* to be ~ 1.7 hours. As I approaches I_{max} , the quantity of $\ln(1-I/I_{max})$ becomes sensitive to small fluctuations in I , resulting in the noisy spikes seen after 6 hours. This portion of the data was not used in the fitting. (C) Estimation of Kaede mRNA stability using *in situ* hybridization. CyA, cyclopamine, an antagonist of Shh signaling that represses *ptch2* promoter output. When soaked in CyA (CyA ctrl), the embryos exhibit a low basal level of Kaede expression in the neural tube. When CyA was added around 9-somite stage and embryos followed over time, the high level of Kaede mRNA as seen in the DMSO control was not maintained but decayed towards the basal level. In the trunk/tail neural tube (but not in the head), the level became similar to basal at +7.5 hours by comparing the CyA and CyA ctrl images. This result suggests Kaede mRNA is around for more than 6 hours after inhibition of signaling, with a half-life of about 3 hours. Scale bar: 400 μ m.

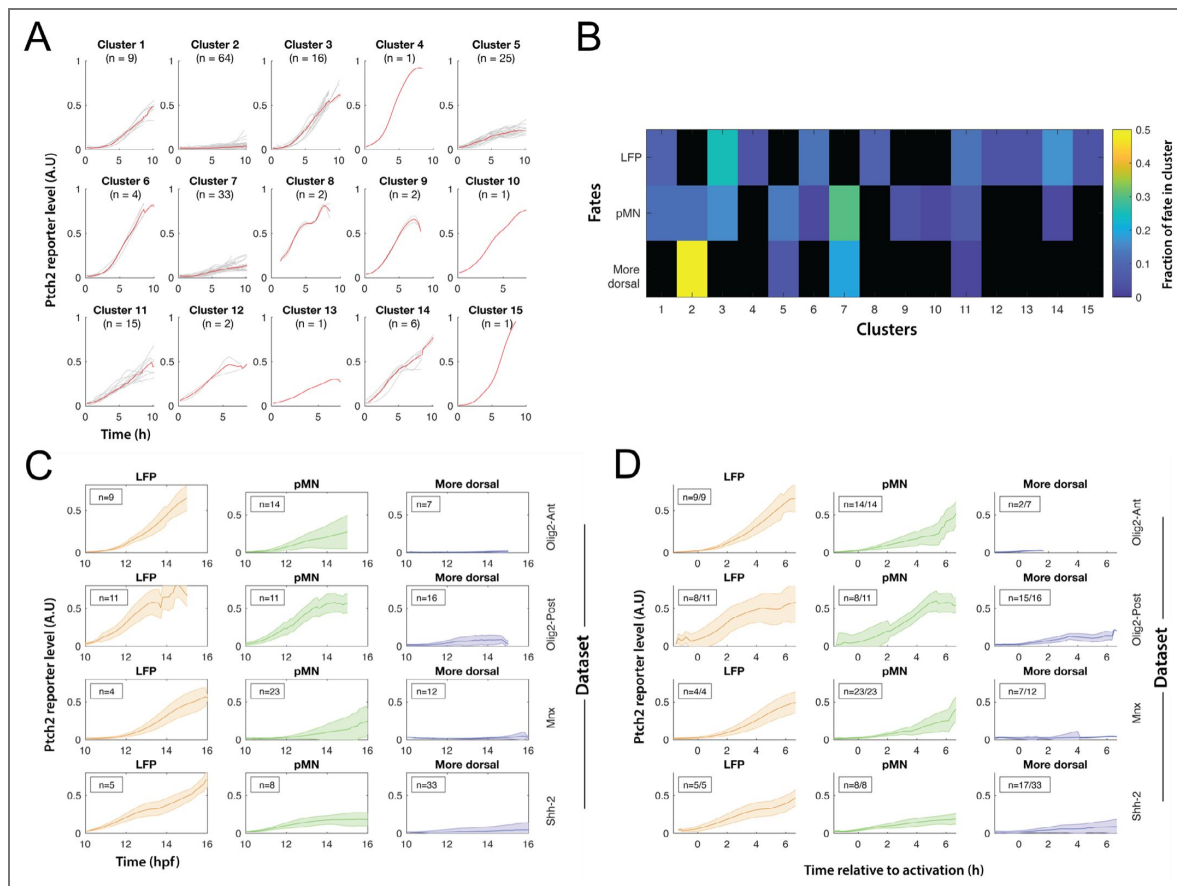


Figure 5. K-means clustering and temporal re-alignment analysis of tracks across datasets.

(A) K-means clustering combining smoothed tracks (as in Figure 3B) from 4 datasets combining *ptch2:kaede* with different reporters (*olig2:gfp*, imaged anteriorly: ‘Olig2-Ant’ or posteriorly: ‘Olig2-Post’; *mnx1:gfp* (‘Mnx’); *shh:gfp* (‘Shh-2’)). (B) Fate contribution of clusters in (A). Heatmap shows, for each fate, the fraction of cells in different clusters. Black indicates no cells. (C) Average *ptch2:kaede* dynamics by cell fate and dataset from processed tracks. (D) Similar to (C), realigned starting times by the first time the modeled *ptch2* promoter output exceeds a threshold of 0.025 of the normalized activity.

correlated with cell fates. For the anterior tracks, the LMDV distance is consistently >90% correlated while for the posterior tracks the LMDV distance is more poorly correlated at earlier stages but becomes better at later times consistent with a necessary role for cell sorting in sharpening fate domain boundaries over time (Xiong et al., 2013 [DOI](#); Tsai et al., 2020 [DOI](#)). In contrast and as expected, the AP distances never correlate with fate choices in the anterior or posterior spinal cord (all around 50%). The diversity of tracks and tissue geometry both within and across the anterior and posterior regions of the spinal cord (Figure 6B [DOI](#)) enables us to search for a common metric that best correlates the Shh response dynamics with fate choice.

We compared the same sets of cell tracks by the maximum transient level, total time, and average levels of Shh response (Figure 7A [DOI](#)), when ventral neural progenitors are known to become specified (Lewis and Eisen, 2003 [DOI](#); Park et al., 2004 [DOI](#); Xiong et al., 2013 [DOI](#)). For the anterior tracks, all metrics are >85% correlative with fate choices, consistent with previous population level studies in amniotes (Ericson et al., 1997 [DOI](#); Dessaud et al., 2007 [DOI](#); Dessaud et al., 2010 [DOI](#)). Interestingly, for the posterior tracks, only maximum transient level correlates with fate choice for >80% of the tracks and the other two metrics fail to make 70%. This difference of correlation percentage is robust to wide variations in values of parameters used in our quantification (e.g. estimated Kaede mRNA half-life, smoothing parameters used in intensity calculation and transcription activity fitting) (Figures 7B-C [DOI](#)). Other metrics (except LMDV cell position at late times, which is one of our fate identifiers) do not correlate better than the maximum transient level when both anterior and posterior tracks are considered (Figure 7D [DOI](#), data not shown). No single metric makes perfect predictions.

Discussion

The imaging, cell tracking, and data analysis reported in this study open a fresh perspective for looking at the important classic problem of morphogen interpretation. While it is reassuring that our data (obtained with a distinct technique) on average corroborate numerous previous studies on Shh mediated ventral neural tube patterning, the increased resolution into single cells and wide temporal coverage from when cells start responding to Shh to cell fate-specification reveal a high degree of previously unrecognized heterogeneity. This single cell variability could be due to other factors that differ between cells and their cross-talk with the Shh gene regulatory network (GRN), such as Notch signaling (Huang et al., 2012 [DOI](#)), or just simply noise that arises in the GRN (Rosenfeld et al., 2005 [DOI](#)). These possibilities are difficult to distinguish with the existing data, but they pose a challenge to morphogen patterning in general: how to ensure pattern precision despite this high heterogeneity in signal responses?

One possibility is to code morphogen response in a robust way under the given heterogeneity. Through quantifying the transcriptional dynamics at the Shh reporter, we are able to directly assess the correlation between certain proposed information-coding schemes and fate outcome. In the anterior spinal cord, maximum transient level, response time, and average level are all predictive of fates. In the more posterior spinal cord however, our results show that the temporal dimension of Shh response (which is part of the response time and the average level) no longer explains cell fate choices as well as the maximum transient level. The cause of this difference might be that there are intrinsic or environmental differences between neural progenitors in the anterior vs. posterior spinal cord, which affect their morphogen interpretation. Supporting this idea, the tissue geometry, and the range and movement of cells in zebrafish neural tube vary greatly along the body axis (Xiong et al., 2013 [DOI](#); this study). We also found that some of the posterior LFP and pMN tracks still show very similar Shh response dynamics even after response initiation time re-alignment, distinct from the tracks from anterior datasets, suggesting an overall noisier environment in the posterior neural tube in zebrafish. This may be because posterior neural progenitors undergo more cell mixing, because their specification times are later or more variable, or because the spatially small Shh gradient as a result of the posterior neural tube's small size introduces more positional noise in signaling. Overall, simple response-fate models are insufficient to account for all the heterogeneities observed or to make precise fate predictions.

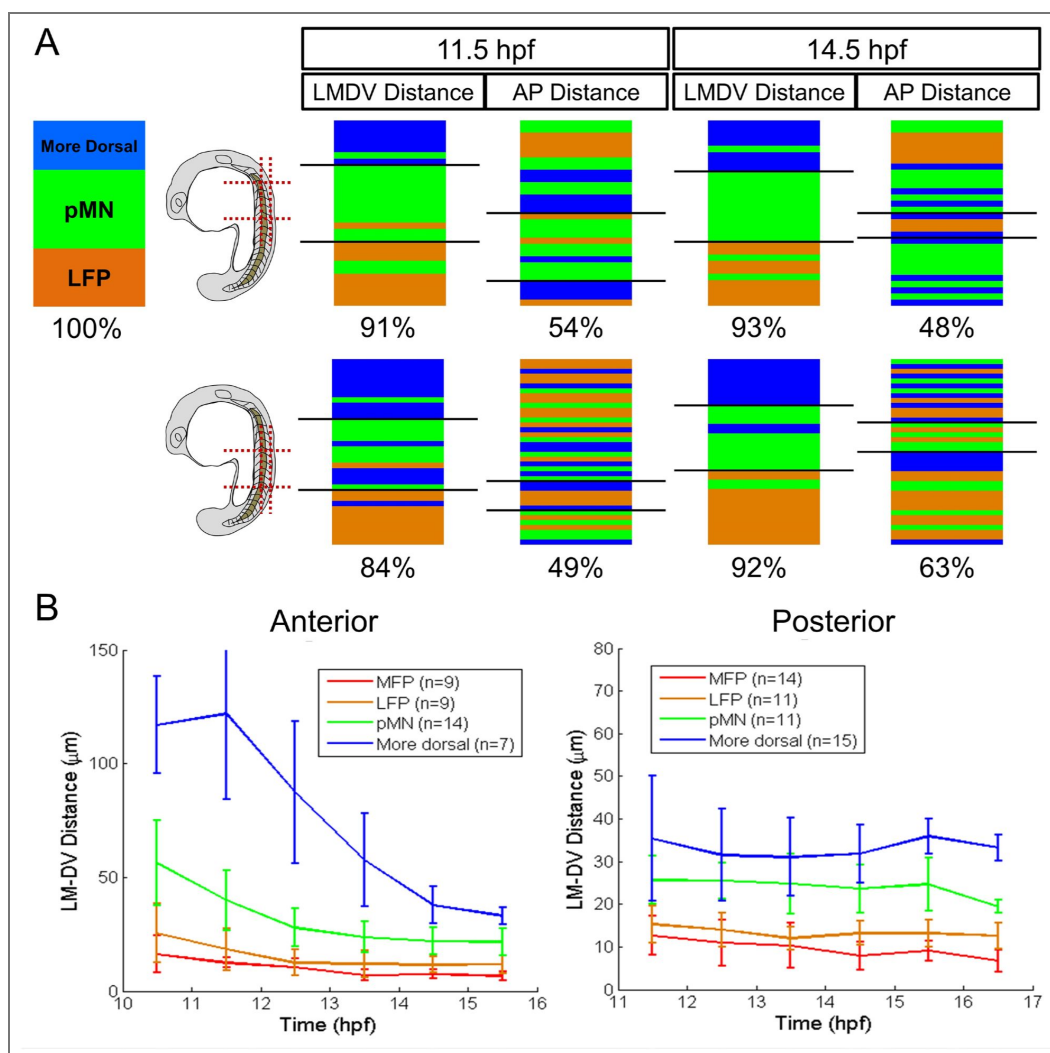


Figure 6. Correlations between position and fate choice in single cells.

(A) Ranking of lateral-medial/dorsal-ventral (LMDV) and anterior-posterior (AP) positions as a control for the correlational analysis. Data from an anterior movie and a posterior movie are compared (schematics). The black lines on the flag mark thresholds that best separate different fates. See descriptions in the text and also methods. (B) Average position tracks. Note the range of anterior progenitors vs. posterior ones over the same time window, indicating different tissue geometry and cell dynamics along the body axis. LMDV: Lateral Medial-Dorsal Ventral distances. Error bars are S.D.

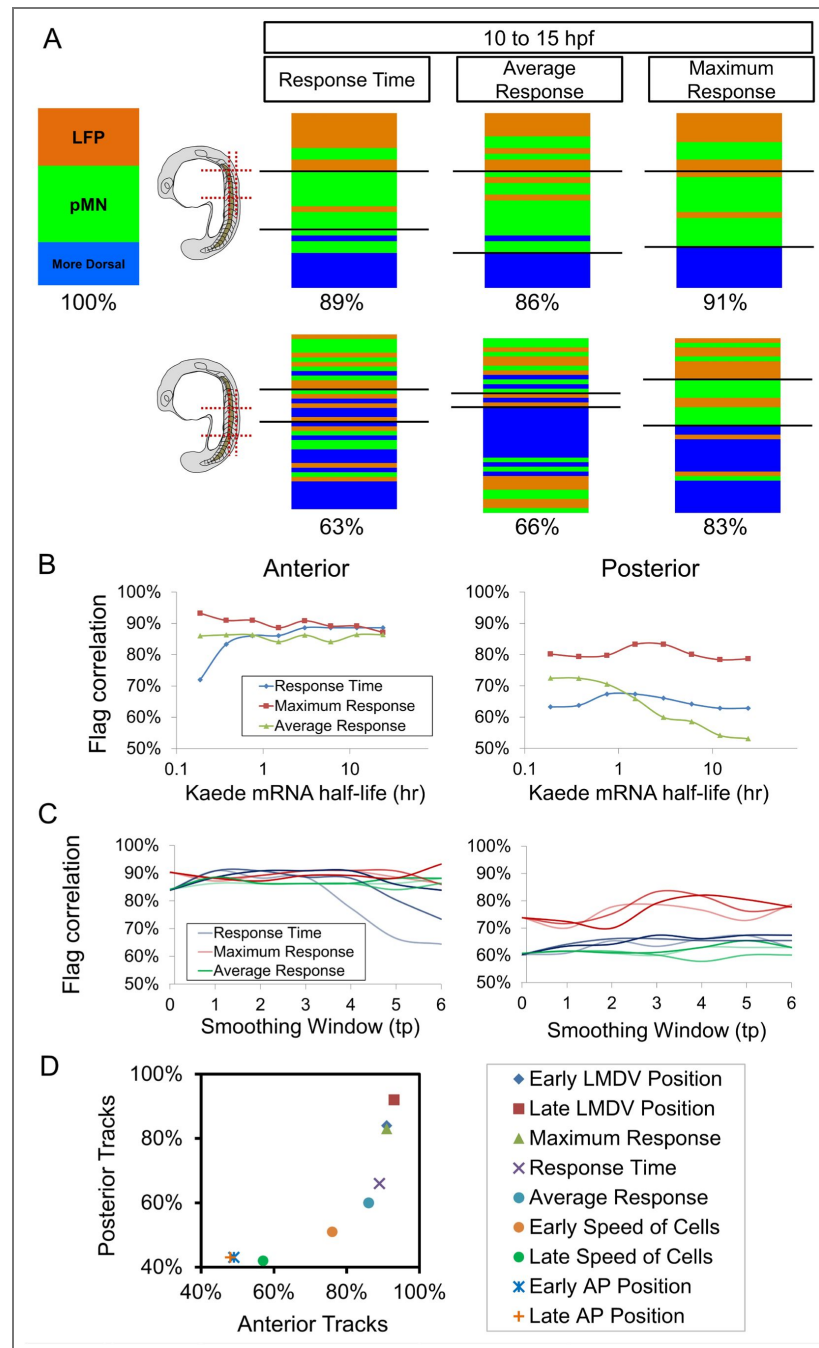


Figure 7. Correlations between different metrics of Shh response and fate choice in single cells.

(A) Shh response dynamics metrics. Under the modeled activity of the *ptch2:kaede* promoter, Response time is a count of time points at which *ptch2:kaede* promoter is “on” (>0.05 A.U./hr²). Average response is the average promoter activity across the whole time window. Maximum response is the highest promoter activity found in the time window. (B) Flag correlation results of the 3 metrics in (A) (for which half-life=3 hours was used) over different Kaede mRNA half-life values. Note that response time and average response parameters switch predictive power as Kaede mRNA becomes stable. Maximum transient response level stays best over wide ranges of mRNA stability. The conclusion in (A) is therefore robust to this estimated parameter (Figure 4). (C) Flag correlation results of the 3 parameters in (A) (where smoothing window=3 time points and iteration number=2) over different smoothing window (x axis) and iteration numbers (dark,1;light,2;lighter,3). The conclusion in (A) is therefore robust to this analysis, despite the sensitivity of equation (3) to the smoothing parameters. (D) Summary of correlation percentages for posterior vs. anterior tracks for different morphogen interpretation models. In addition to metrics mentioned in the main text, cell speeds were also plotted here. The speeds were calculated from the positions of the cells in the track for 2-hour window centered on the early (11.5hpf) and late (14.5hpf) time points.

While it is tempting to construct more complex models using our single cell data, certain limitations remain that warrant caution. First, our live imaging is limited by the brightness of reporters and the sensitivity of detection. Dynamics that fall below our imaging capacity, especially the Shh response activities before and in the early hours of our tracks, are not as well resolved, such that the initiation time of progenitor response and its heterogeneity may not be captured. Second, due to the constraint of a live embryo undergoing morphogenesis, certain cells cannot be fully tracked throughout the imaging time window. Third, even though most ventral cells are specified and no longer require Shh by the end of our tracks (17–18hpf, Huang et al., 2012 [↗](#); Xiong et al., 2013 [↗](#)), some cells may remain unspecified and their fate reporter could further change beyond our detection. These limitations could give misleading results in overly fine-grained comparisons (e.g., response level within each 1-hour window, data not shown), where a much smaller number of tracks and time points could dominate and thus bias the analysis.

Nevertheless, the results presented here along with our previous study (Xiong et al., 2013 [↗](#)) raise an important consideration for the understanding of morphogen interpretation: given the pervasive heterogeneity of single cells in position, timing, and signaling, how is patterning precision achieved? We speculate that precision at the single cell level may not be necessary as the heterogeneity can average out on the population level, and thus important patterning features such as the size of each progenitor domain can remain robust even under single cell noise. Still, it is important to note that the Shh GRN examined here already provides a strong degree of accuracy in single cells (e.g., Shh maximum response to fate choices) across datasets and explains a large part of the final pattern as seen on the population level. An additional morphogen gradient such as BMP from the dorsal neural tube could provide information in parallel with Shh to improve fate choice precision as a function of position (Zagorski et al., 2017 [↗](#)), which is inherently challenging for a single morphogen gradient to convey (Zagorski et al., 2024 [↗](#)). After cell fate choices were made, remaining positional errors can be corrected by cellular mechanisms such as cell sorting to refine the final pattern (Tsai et al., 2020 [↗](#)). Finally, additional mechanisms such as apoptosis may provide means of correction. Further testing of these ideas would require improved imaging capacities and access to multiple nodes of the GRN with more accurate live reporters (e.g., fluorescent protein knock-ins). Larger-scale, more systematic single-cell imaging and analysis will allow a finer and potentially definitive quantitative dissection of this question.

Materials and methods

Zebrafish strains and maintenance

Tg(shh:gfp) (Shkumatava et al., 2004 [↗](#)), *tgBAC(ptch2:kaede)* (Huang et al., 2012 [↗](#)), *tg(nkx2.2a:mgfp)* (Ng et al., 2005 [↗](#)), *tg(olig2:gfp)* (Shin et al., 2003 [↗](#)), *tg(mnx1:gfp)* (Flanagan-Steet et al., 2005 [↗](#)) and *tg(hsp70l:EGFP)* (Halloran et al., 2000 [↗](#)) have been described. Homozygote parents from two different lines were paired to produce double transgenic embryos for imaging. Natural spawning was used and time of fertilization was recorded at the single cell stage of each clutch. Embryos were kept in 28°C incubators/chambers during imaging and other times except room temperature during injections, dechorionating and mounting. Staging was recorded using morphological criteria and aligned to the normal table (Kimmel et al., 1995 [↗](#)). All fish are housed in fully equipped and regularly maintained and inspected aquarium facilities. Fish-related protocols have been approved by the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School.

Microinjections of mRNAs

A pMTB construct (Xiong et al., 2013 [↗](#)) containing *mem-EBFP2* or *h2b-EBFP2* was used for mRNA synthesis with the mMESSAGE mMACHINE system (Ambion). For mosaic injections, one blastomere of 8 to 16-cell stage embryos was injected (Nanoject) with approximately 1nl of 20ng/μl *mem-EBFP2* or *h2b-EBFP2*. One round of screening was applied immediately after mosaic injections to eliminate damaged embryos and/or ones that missed the injection (no retention of co-injected Phenol Red label). Before imaging, injected embryos were screened for health.

Timelapse confocal imaging

Live imaging was performed using a Zeiss 710 confocal microscope (objective: C-Apochromat 40X 1.2 NA) with a home-made heating chamber maintaining 28°C. Embryos were mounted using the dorsal mount (Megason 2009 [↗](#)) with a stereoscope (Leica MZ12.5). The mounting steps for imaging the neural plate/tube have been described in detail in Xiong et al., 2013 [↗](#). 405nm laser was used for photo conversion of Kaede and excitation of EBFP2 at the same time. 488nm and 561nm were then used in a separate track for the GFP and Kaede(Red) signals (See also Figure 1B [↗](#)). A typical stack covers an imaging space of 303µm(x), 303µm(y) and 120µm(z), divided by 700X700X80 voxels and is taken within 6 minutes. A typical movie lasts ~10 hours containing ~100 stacks at 6 minute intervals.

Data analysis

Track creation and fate assignment

For the present study 31 timelapse datasets were collected (29 contain *ptch2:kaede* with *olig2:gfp*:15; *nkx2.2:mgfp*:6; *shh:gfp*:4; *mnx1:gfp*:4. 2 contain *olig2:dsRed* and *nkx2.2:mgfp*). 21/31 allow cell tracking and 13/21 have high coverage. Tracks were generated for 7/13 and 4/7 were further processed. The datasets acquired in Zeiss .lsm format were first converted to Megacapture format for import into GoFigure2 software (www.gofigure2.org [↗](#), see also Xiong et al., 2013 [↗](#); Xiong et al., 2014 [↗](#)). Inside GoFigure2, segmentation and tracking were performed manually on each dataset on randomly selected ventral neural tube cells in the labeled embryo (e.g., Figures 1D-E [↗](#)). To calculate the cell's positions in relation to the embryo, notochord segmentations were generated every 10 time points (1 hour in real time) for each presented dataset. Segmentation and track tables exported from GoFigure2 were further processed using custom scripts (provided in accompanying supplemental data files) to generate the single slice labeled movies (Movie S2 [↗](#)). The movies were watched individually to determine the fate choice of the cell using a combinatorial criteria including fate reporter expression and final position in the ventral neural tube, as described in Xiong et al., 2013 [↗](#). First, at 16hpf, MFPs were identified as the centered, apically constricted, triangle-shaped column of cells immediately dorsal to the notochord (as illustrated in Figure 1A [↗](#)). This fate identification is checked with *shh:gfp* movies in which MFPs are GFP+. Next, LFPs are identified as the two columns of cells flanking both sides of the MFP (Huang et al., 2012 [↗](#)). This fate identification is confirmed with *nkx2.2:mgfp* movies (e.g., Movie S1 [↗](#)). Next, pMNs are identified as the cells dorsal to the floor plate cells that are strongly *olig2:gfp* positive. Finally, More dorsal cells are those that are further dorsal to the pMN domain and GFP- in *olig2:gfp* movies or that are located >40% ventral dorsal distance at 16hpf in movies without *olig2:gfp* signal. The segmentations, tracks and cell fate information were then assembled through a preprocessing pipeline in Matlab (Mathworks).

Track processing

The output of the pipeline is a set of Matlab matrixes containing organized raw data for downstream analysis (e.g., *NewFinalWrkSpace_olig2_9.mat*, see supplemental data files). This organized raw data was then processed through a series of filters (provided in accompanying supplemental data files) to account for multiple experimental factors that introduce small errors in intensity measurements including bleed-through, saturation, and noise (e.g. *SaturationMask.m*: a first filter that removes aberrations in intensity calculation arising from saturated pixels in the image. *BleedthroughCorr.m*: correcting bleed-through signal between two channels. Bleed-through correction runs automatically after the algorithm assesses the degree of bleed-through and then applies a percentage subtraction on the values to further reduce errors in intensity calculation. *SmoothData.m*: a smoothing filter that handles short-time fluctuations that come from segmentation variations and acquisition noise. Different utility scripts were coded to allow user interactions with both the raw and filtered data, listed in *AnalysisScript_List.xlsx*.

Track alignment

ptch2:kaede reporter tracks were aligned at the point at which the corresponding promoter activity traces crossed an activation threshold value and maintained it for three timepoints (18 min). The activation threshold was determined by comparing promoter activity values from more dorsally-fated cells, in which the reporter is generally inactive, and those from LFP- and pMN-fated cells, in which the reporter is activated. A threshold value of 0.025 could distinguish the two sets of traces, with only ~60% of more dorsally-fated cells crossing the threshold at any point within the observation period compared to 100% of LFP- and pMN-fated cells.

Track clustering analysis

ptch2:kaede reporter traces were clustered using the k-means clustering function in MATLAB. Each track was truncated to the set of timepoints for which >80% of reporter traces had observations. After truncation, tracks were only included in the analysis if they had observations for >80% of remaining timepoints. After filtering, 182 out of 216 traces were included in the clustering analysis. The elbow method was used to determine an appropriate number of clusters. The number of clusters was varied from 5 to 25, and for each value, the total Within Cluster Sum of Squares (WCSS) value was calculated. For each cluster the WCSS value corresponds to the sum of square distances for each point in the cluster from the centroid of the cluster. The total WCSS value decreases with an increasing number of clusters (because individual clusters become tighter) and begins to plateau at approximately 15 clusters. This value was therefore used for the analysis.

Data availability

Full analysis protocols, scripts and sample datasets are available for download here:
https://github.com/xionglab/NT_heterogeneity 

Acknowledgements

We thank D. D'India for fish care, K. Mosaliganti, T. Tsai and members of Matlab community for sharing scripts, A. Green, B. Appel and J. Kuwada for transgenic lines, W. Ma, T. Mitchison, A. Schier, and Megason lab members for discussions. This work is supported by NIH R01 GM107733 to S.G.T.M. and Natural Sciences and Engineering Research Council of Canada (NSERC) to P.H. F.X. also acknowledges the support of a UKRI-EP SRC Frontier Research Grant (EP/X023761/1, originally selected as an ERC Starting Grant).

Additional information

Author Contributions

F.X., A.R.T. and S.G.T.M. conceived this study. F.X. and A.R.T. performed the experiments and analyzed the data. S.N. performed track clustering and variability analysis. T.W.H. performed *hsp70l:EGFP* experiments and contributed to data analysis. P.H. contributed data of *ptch2:kaede* line. F.X. and S.G.T.M. prepared the manuscript with input from all authors. The authors declare no competing financial interest.

Funding

Funder	Grant reference number	Author
HHS National Institutes of Health (NIH)	GM107733	Sean G Megason
UKRI Engineering and Physical Sciences Research Council (SRC)	EP/X023761/1	Fengzhu Xiong

Author ORCID iDs

Fengzhu Xiong: <https://orcid.org/0000-0002-6153-0254>

Sean G Tsung-Megason: <https://orcid.org/0000-0002-9330-2934>

Additional files

Movie S1 [h2b-ebfp2, ptch2:kaede/nkx2.2:mgfp](#) dataset. 3D rendered maximum projections. Top view and cross-sectional view. Nuclear EBFP2 label allows easier tracking and identification of *nkx2.2:mgfp* cells.

Movie S2 [Slice movie showing a single pMN track in a mem-ebfp2, ptch2:kaede/mnx1:gfp](#) dataset. Using a track as input, the movie was automatically generated by re-slicing the dataset centering on the tracked cells (marked by the white dot). The slices thereby allow visual observation of the cell's behavior even as it changes position. This cell is seen to move towards the midline and turn on the GFP reporter, adopting a pMN fate. Top view and cross-sectional view. Time stamp: hh:mm. Scale bar: 10µm. Same below.

Movie S3 [mem-ebfp2, ptch2:kaede/olig2:gfp](#) anterior dataset. Anterior neural tube formation. Epiboly movement and convergence are apparent in the beginning of the movie.

Movie S4 [mem-ebfp2, ptch2:kaede/olig2:gfp](#) posterior dataset. Posterior neural tube formation. Blastopore closure is apparent in the beginning of the movie. Tissue is overall smaller than the anterior counterpart ([Movie S3](#)).

References

- Ando R., Hama H., Yamamoto-Hino M., Mizuno H., Miyawaki A. (2002) An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A* **99**:12651-12656
- Balaskas N., Ribeiro A., Panovska J., Dessaud E., Sasai N., Page K.M., Briscoe J., Ribes V. (2012) Gene regulatory logic for reading the Sonic Hedgehog signaling gradient in the vertebrate neural tube. *Cell* **148**:273-284
- Briscoe J., Pierani A., Jessell T.M., Ericson J. (2000) A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101**:435-445
- Dessaud E., Yang L.L., Hill K., Cox B., Ulloa F., Ribeiro A., Mynett A., Novitch B.G., Briscoe J. (2007) Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism. *Nature* **450**:717-720
- Dessaud E., Ribes V., Balaskas N., Yang L.L., Pierani A., Kicheva A., Novitch B.G., Briscoe J., Sasai N. (2010) Dynamic assignment and maintenance of positional identity in the ventral neural tube by the morphogen sonic hedgehog. *PLoS Biol* **8**:e1000382
- Driever W., Nüsslein-Volhard C. (1989) The bicoid protein is a positive regulator of hunchback transcription in the early Drosophila embryo. *Nature* **337**:138-143
- Driever W., Thoma G., Nüsslein-Volhard C. (1989) Determination of spatial domains of zygotic gene expression in the Drosophila embryo by the affinity of binding sites for the bicoid morphogen. *Nature* **340**:363-367
- Elowitz M.B., Leibler S. (2000) A synthetic oscillatory network of transcriptional regulators. *Nature* **403**:335-338
- Ericson J., Rashbass P., Schedl A., Brenner-Morton S., Kawakami A., van Heyningen V., Jessell T.M., Briscoe J. (1997) Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**:169-180
- Flanagan-Steet H., Fox M.A., Meyer D., Sanes J.R. (2005) Neuromuscular synapses can form in vivo by incorporation of initially aneural postsynaptic specializations. *Development* **132**:4471-4481
- Halloran M.C., Sato-Maeda M., Warren J.T., Su F., Lele Z., Krone P.H., Kuwada J.Y., Shoji W. (2000) Laser-induced gene expression in specific cells of transgenic zebrafish. *Development* **127**:1953-1960
- Huang P., Xiong F., Megason S.G., Schier A.F. (2012) Attenuation of Notch and Hedgehog signaling is required for fate specification in the spinal cord. *PLoS Gen* **8**:e1002762

- Jeong J., McMahon A.P. (2005) Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1. *Development* **132**:143-154
- Jessell T.M. (2000) Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet* **1**:20-29
- Kicheva A, Briscoe J. (2023) Control of Tissue Development by Morphogens. *Annu Rev Cell Dev Biol* **39**:91-121
- Kimmel C., Ballard W., Kimmel S., Ullmann B., Schilling T. (1995) Stages of embryonic development of the zebrafish. *Dev. Dyn* **203**:253-310
- Lek M., Dias J.M., Marklund U., Uhde C.W., Kurdija S., Lei Q., Sussel L., Rubenstein J.L., Matisse M.P., Arnold H.H., et al. (2010) A homeodomain feedback circuit underlies step-function interpretation of a Shh morphogen gradient during ventral neural patterning. *Development* **137**:4051-4060
- Lewis K.E., Eisen J.S. (2003) From cells to circuits: development of the zebrafish spinal cord. *Prog. Neurobiol* **69**:419-449
- Megason S. (2009) Methods in Molecular Biology. *Methods Mol Biol* **546**:317-332
- Ng A.N., de Jong-Curtain T.A., Mawdsley D.J., White S.J., Shin J., Appel B., Dong P.D., Stainier D.Y., Heath J.K. (2005) Formation of the digestive system in zebrafish: III. Intestinal epithelium morphogenesis. *Dev. Biol* **286**:114-135
- Oosterveen T., Kurdija S., Alekseenko Z., Uhde C.W., Bergsland M., Sandberg M., Andersson E., Dias J.M.M., Muhr J., Ericson J. (2012) Mechanistic differences in the transcriptional interpretation of local and long-range Shh morphogen signaling. *Dev. Cell* **23**:1006-1019
- Park H.C., Shin J., Appel B. (2004) Spatial and temporal regulation of ventral spinal cord precursor specification by Hedgehog signaling. *Development* **131**:5959-5969
- Rieger T. R., Morimoto R. I., Hatzimanikatis V. (2005) Mathematical modeling of the eukaryotic heat-shock response: dynamics of the hsp70 promoter. *Biophysical journal* **88**:1646-1658
- Rosenfeld N., Young J.W., Alon U., Swain P.S., Elowitz M.B. (2005) Gene regulation at the single-cell level. *Science* **307**:1962-1965
- Shin J., Park H.-C., Topczewska J.M., Mawdsley D.J., Appel B. (2003) Neural cell fate analysis in zebrafish using olig2 BAC transgenics. *Meth Cell Sci* **25**:7-14
- Shkumatava A., Fischer S., Muller F., Strahle U., Neumann C.J. (2004) Sonic hedgehog, secreted by amacrine cells, acts as a short-range signal to direct differentiation and lamination in the zebrafish retina. *Development* **131**:3849-3858
- Tsai T.Y.C., Sikora M., Xia P., Colak-Champollion T., Knaut H., Heisenberg C.P., Megason S.G. (2020) An adhesion code ensures robust pattern formation during tissue morphogenesis. *Science* **370**:113-116
- Wolpert L. (1969) Positional information and the spatial pattern of cellular differentiation. *J. Theor. Biol* **25**:1-47
- Xiong F., Ma W., Hiscock T.W., Mosaliganti K.R., Tentner A.R., Brakke K.A., Rannou N., Gelas A., Souhait L., Swinburne I.A., et al. (2014) Interplay of cell shape and division orientation promotes robust morphogenesis of developing epithelia. *Cell* **159**:415-427
- Xiong F., Megason S.G. (2015) Abstracting the principles of development using imaging and modeling. *Integr Biol* **7**:633-642
- Xiong F., Tentner A.R., Huang P., Gelas A., Mosaliganti K.R., Souhait L., Rannou N., Swinburne I.A., Obholzer N.D., Cowgill P.D., et al. (2013) Specified neural progenitors sort to form sharp domains after noisy Shh signaling. *Cell* **153**:550-561
- Zagorski M., Tabata Y., Brandenberg N., Lutolf M. P., Tkačik G., Bollenbach T., ..., Kicheva A. (2017) Decoding of position in the developing neural tube from antiparallel morphogen gradients. *Science* **356**:1379-1383

Zagorski M., Brandenberg N., Lutolf M., Tkacik G., Bollenbach T., Briscoe J., Kicheva A. (2024) Assessing the precision of morphogen gradients in neural tube development. *Nature Communications* **15**:929

Peer reviews

Reviewer #1 (Public Review):

[Editors' note: This version has been assessed by the Reviewing Editor without further input from the original reviewers. Given the time elapsed since the original data collection, the authors have addressed the previous concerns by providing a more nuanced discussion of their results and acknowledging the limitations of the study to ensure the conclusions are supported by the existing data.]

Throughout the paper, the authors do a fantastic job of highlighting caveats in their approach, from image acquisition to analysis. Despite this, some conclusions and viewpoints portrayed in this study do not appear well-supported by the provided data. Furthermore, there are a few technical points regarding the analysis that should be addressed.

(1) Analysis of signaling traces

- Relevance of "modeled signaling level": It is not clear whether this added complexity and potential for error (below) provides benefits over a more simple analysis such as taking the derivative (shown in Figure 3C). Could the authors provide evidence for the benefits? For example, does the "maximal response" given a simpler metric correlate less well with cell fate than that calculated from the fitted response?

- Assumptions for "modeled signaling level": According to equation (1) Kaede levels are monotonically increasing. This is assumed given the stability of the fluorescent protein. However, this only holds for the "totally produced Kaede/fluorescence". Other metrics such as mean fluorescence can very well decrease over time due to growth and division. Does "intensity" mean total fluorescence? Visual inspection of the traces shown in Figure 2 suggests that "fluorescence intensity" can decrease. What does this mean for the inferred traces?

- Estimation of Kaede reporter half-life: It is not clear how the mRNA stability of Kaede is estimated. It sounds like it was just assessed visually, which seems not entirely appropriate given the quantitative aspects of the rest of the study. Also, given that Shh signaling was inhibited on the level of Smoothed, it is not obvious how the dynamics of signaling shutdown affect the estimate. Most results in Figure 7 seem to be quite robust to the estimate of the half-life. That they are, might suggest that the whole analysis is unnecessary in the first place. However, not all are. Thus, it would be important to make this estimate more quantitative.

(2) Assignment of fates and correlations

- Error estimate for cell-type assignment: Trying to correlate signaling traces to cell fate decisions requires accurate cell fate assignment post-tracking. The provided protocol suggests a rather manual, expert-directed process of making those decisions. Can the authors provide any error-bound on those decisions, for example comparing the results obtained by two experts or something comparable? I am particularly concerned about the results regarding the higher degree of variability in the correlation between signaling dynamics and cell fate in the posterior neural tube. Here, the expression of Olig2 does not seem to segregate between different assigned fates, while it does so nicely in the anterior neural tube. This would suggest to me that cells in the posterior neural tube might not yet be fully committed to a fate or that there could be a relatively high error rate in assigning fates. Thus, the results could emerge

from technical errors or differences in pure timing. Could the authors please comment on these possibilities?

- Clustering and fates: One approach the authors use to analyze the correlation between signaling and fate is clustering of cell traces and comparison of the fate distributions in those clusters. There is a large number of clusters with only single traces, suggesting that the data (number of traces) might not be sufficient for this analysis. Furthermore, I am skeptical about clustering cells of different anterior-posterior identities together, given potential differences in the timing of signal reception and signaling. I am not convinced that this analysis reveals enough about how signaling maps to fate given the heterogeneity in traces in large clusters and the prevalence of extremely small clusters.

- Signaling vector and hand-picked metrics: As an alternative approach, that might be better suited for their data, the authors then pick three metrics (based on their model-predicted signaling dynamics) and show that the maximal response is a very good predictor of fate for different anterior-posterior identities. Previous information-theoretic analysis of signaling dynamics has found that a whole time-vector of signaling can carry much more information than individual metrics (Selimkhanov et al, 2014, PMID: 25504722). Have the authors tried to use approaches that make use of the whole trace (such as simple classifiers (Granados et al, 2018, PMID: 29784812), or can comment on why this is not feasible for their data? The authors should at least make clear that their results present a lower bound to how accurately cells can make cell-fate decisions based on signaling dynamics.

(3) Consequences of signaling heterogeneity

The authors focus heavily on portraying that signaling dynamics are highly variable, which seems visually true at first glance. However, there is no metric used or a description given of what this actually means. Mainly, the variability seems to relate to the correlation between signaling and fate. However, given the data and analysis, I would argue that the decoding of signaling dynamics into fate is surprisingly accurate. So signaling dynamics that seem quite noisy and variable by visual inspection can actually be very well discriminated by cells, which to me appears very exciting.

Indeed, simple features of signaling traces can predict cell fate as well as position (for anterior progenitors). Given that signaling should be a function of position, it naively seems as if signaling read-out could be almost perfect. It might be interesting to plot dorsal-ventral position vs the signaling metrics, to also investigate how Shh concentration/position maps to signaling dynamics, this would give an even more comprehensive view of signal transmission.

There remains the discrepancy between signaling traces and fate in the posterior neural tube. The authors point towards differences in tissue architecture and difficulties in interpreting a "small" Shh gradient. However, the data seems consistent with differences in timing of cell-fate decisions between anterior and posterior cells. The authors show that fate does initially not correlate well with position in the posterior neural tube. So, signaling dynamics should likely also not, as they should rather be a function of position, given they are downstream of the Shh gradient. As mentioned above, not even Olig2 expression does segregate the assigned fates well. All this points towards a difference in the time of fate assignment between the anterior and posterior. Given likely delays in reporter protein production and maturation, it can thus not be expected that signaling dynamics correlate better with cell fate than the reporter "83%". Can the authors please discuss this possibility in the paper?

Thus, while this paper represents an example of what the community needs to do to gain a better understanding of robust patterning under variability, the provided data is not always sufficient to make clear conclusions regarding the functional consequences of signaling dynamics.

<https://doi.org/10.7554/eLife.96980.2.sa2>

Reviewer #2 (Public Review):

Summary:

In this work, Xiong and colleagues examine the relationship between the profile of the morphogen Shh and the resulting cell fate decisions in the zebrafish neural tube. For this, the authors combine high-resolution live imaging of an established Shh reporter with reporter lines for the different progenitor types arising in the forming neural tube. One of the key observations in this manuscript is that, while, on average, cells respond to differences in Shh activity to adopt distinct progenitor fates, at the single cell level there is strong heterogeneity between Shh response and fate choices. Further, the authors showed that this heterogeneity was particularly prominent for the pMN fate, with similar Shh response dynamics to those observed in neighboring LFP progenitors.

Strengths:

It is important to directly correlate Shh activity with the downstream TFs marking distinct progenitor types in vivo and with single cell resolution. This additional analysis is in line with previous observations from these authors, namely in Xiong, 2013. Further, the authors show that cells in different anterior-posterior positions within the neural tube show distinct levels of heterogeneity in their response to Shh, which is a very interesting observation and merits further investigation.

Weaknesses:

This is a convincing work, however, adding a few more analyses and clarifications would, in my view, strengthen the key finding of heterogeneity between Shh response and the resulting cell fate choices.

<https://doi.org/10.7554/eLife.96980.2.sa1>

Author Response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

Throughout the paper, the authors do a fantastic job of highlighting caveats in their approach, from image acquisition to analysis. Despite this, some conclusions and viewpoints portrayed in this study do not appear well-supported by the provided data. Furthermore, there are a few technical points regarding the analysis that should be addressed.

We thank the reviewer for the comments, due to the age of the work and logistic constraints, we are unable to perform further experiments and analysis to address some of the concerns. We revised conclusions and viewpoints accordingly to reflect reviewer concerns.

(1) Analysis of signaling traces

Relevance of "modeled signaling level": It is not clear whether this added complexity and potential for error (below) provides benefits over a more simple analysis such as taking the derivative (shown in Figure 3C). Could the authors provide evidence for the benefits?

For example, does the "maximal response" given a simpler metric correlate less well with cell fate than that calculated from the fitted response?

We think the benefits of modeled signaling level are the conceptual accuracy to the extent possible with the data. It's true that the assumptions brought-in may cause certain biases. We perform this and the simplest (raw data averaging, Fig.2). Intermediate results in between (such as the first derivative in Fig.3C) may correlate well or less well, but cannot be interpreted biologically.

Assumptions for "modeled signaling level": According to equation (1) Kaede levels are monotonically increasing. This is assumed given the stability of the fluorescent protein. However, this only holds for the "totally produced Kaede/fluorescence." Other metrics such as mean fluorescence can very well decrease over time due to growth and division. Does "intensity" mean total fluorescence? Visual inspection of the traces shown in Figure 2 suggests that "fluorescence intensity" can decrease. What does this mean for the inferred traces?

Yes the segmentations measure intensity in a fixed volume inside a cell, therefore it's a spatial average (concentration) and is susceptible to cell volume changes. This has been noted in the revision. The raw measurement does fluctuate and can decrease, we think the short-time-scale fluctuations are likely measurement variations/errors rather than underlying big changes in concentration.

Estimation of Kaede reporter half-live: It is not clear how the mRNA stability of Kaede is estimated. It sounds like it was just assessed visually, which seems not entirely appropriate given the quantitative aspects of the rest of the study. Also, given that Shh signaling was inhibited on the level of Smoothened, it is not obvious how the dynamics of signaling shutdown affect the estimate. Most results in Figure 7 seem to be quite robust to the estimate of the half-live. That they are, might suggest that the whole analysis is unnecessary in the first place. However, not all are. Thus, it would be important to make this estimate more quantitative.

Yes we agree. Unfortunately we don't have the quantitative data required to better estimate Kaede mRNA stability. The timing of Cyc inhibition to the ceasing of ptch mRNA production is roughly estimated but not necessarily precise in this context.

(2) Assignment of fates and correlations

Error estimate for cell-type assignment: Trying to correlate signaling traces to cell fate decisions requires accurate cell fate assignment post-tracking. The provided protocol suggests a rather manual, expert-directed process of making those decisions. Can the authors provide any error-bound on those decisions, for example comparing the results obtained by two experts or something comparable? I am particularly concerned about the results regarding the higher degree of variability in the correlation between signaling dynamics and cell fate in the posterior neural tube. Here, the expression of Olig2 does not seem to segregate between different assigned fates, while it does so nicely in the anterior neural tube. This would suggest to me that cells in the posterior neural tube might not yet be fully committed to a fate or that there could be a relatively high error rate in assigning fates. Thus, the results could emerge from technical errors or differences in pure timing. Could the authors please comment on these possibilities?

This is a very insightful point. We did examine the posterior data again (cross-checked by 2 co-authors) to make sure the mixed situation has correct cell fate assignment. As established by others' and our previous studies (See also Fig.1A), the identification of MFPs and LFPs in zebrafish spinal cord is very robust. The MFPs are the apical constricted single column of cells along the midline on top of the notochord, and the LFPs are the 2 columns of cells next

to MFP on both sides. LFPs' expression of *olig2:gfp* did vary more in the posterior (timing of response/commitment could be a factor as the reviewer pointed out), but eventually the cells at those positions will be V3 interneurons or floor plates and have not been observed to make motoneurons. There are 3 low *Olig2:GFP* pMNs in the anterior dataset (Fig.2B') and 3 high *Olig2:GFP* LFPs in the posterior dataset (Fig.2D') that we checked carefully. The heterogeneity argument is based on the verified tracking and final positioning of these cells.

Clustering and fates: One approach the authors use to analyze the correlation between signaling and fate is clustering of cell traces and comparison of the fate distributions in those clusters. There is a large number of clusters with only single traces, suggesting that the data (number of traces) might not be sufficient for this analysis. Furthermore, I am skeptical about clustering cells of different anterior-posterior identities together, given potential differences in the timing of signal reception and signaling. I am not convinced that this analysis reveals enough about how signaling maps to fate given the heterogeneity in traces in large clusters and the prevalence of extremely small clusters.

We agree. Due to the age of the work and logistic constraints, we are unable to perform further experiments and analysis to enrich the tracks for this revision. We are aware of upcoming, independent studies with many more systematic tracks and analysis which will address these concerns. We have added the caveats the reviewer raised.

Signaling vector and hand-picked metrics: As an alternative approach, that might be better suited for their data, the authors then pick three metrics (based on their model-predicted signaling dynamics) and show that the maximal response is a very good predictor of fate for different anterior-posterior identities. Previous information-theoretic analysis of signaling dynamics has found that a whole time-vector of signaling can carry much more information than individual metrics (Selimkhanov et al, 2014, PMID: 25504722). Have the authors tried to use approaches that make use of the whole trace (such as simple classifiers (Granados et al, 2018, PMID: 29784812), or can comment on why this is not feasible for their data? The authors should at least make clear that their results present a lower bound to how accurately cells can make cell-fate decisions based on signaling dynamics.

Thanks for these suggestions. We are limited by the measurement noise, coverage window of the traces and the number of tracks to make use of the full dynamics in a more informative manner.

(3) Consequences of signaling heterogeneity

The authors focus heavily on portraying that signaling dynamics are highly variable, which seems visually true at first glance. However, there is no metric used or a description given of what this actually means. Mainly, the variability seems to relate to the correlation between signaling and fate. However, given the data and analysis, I would argue that the decoding of signaling dynamics into fate is surprisingly accurate. So signaling dynamics that seem quite noisy and variable by visual inspection can actually be very well discriminated by cells, which to me appears very exciting.

Yes – we agree that most cells are actually accurate in such a highly dynamic tissue. In the literature, the view has been more focused on how the GRN enables this accuracy. We therefore highlighted the heterogeneity and limit of accuracy of the GRN here. We added this point to make our presentation more balanced.

*Indeed, simple features of signaling traces can predict cell fate as well as position (for anterior progenitors). Given that signaling should be a function of position, it naively seems as if signaling read-out could be almost perfect. It might be interesting to plot dorsal-ventral position vs the signaling metrics, to also investigate how *Shh**

concentration/position maps to signaling dynamics, this would give an even more comprehensive view of signal transmission.

We'd refer readers to our earlier study Xiong et al., 2013 where *ptch2:kaede*, *nkx2:gfp* and *olig2:gfp* were plotted against position over time in single cell tracks. It was found that position was not a good predictor of signaling levels or cell fates at early stages when the cell fates were specified.

There remains the discrepancy between signaling traces and fate in the posterior neural tube. The authors point towards differences in tissue architecture and difficulties in interpreting a "small" Shh gradient. However, the data seems consistent with differences in timing of cell-fate decisions between anterior and posterior cells. The authors show that fate does initially not correlate well with position in the posterior neural tube. So, signaling dynamics should likely also not, as they should rather be a function of position, given they are downstream of the Shh gradient. As mentioned above, not even Olig2 expression does segregate the assigned fates well. All this points towards a difference in the time of fate assignment between the anterior and posterior. Given likely delays in reporter protein production and maturation, it can thus not be expected that signaling dynamics correlate better with cell fate than the reporter "83%". Can the authors please discuss this possibility in the paper?

Yes this is an important point/caveat of live signaling and fate tracking. As discussed in the manuscript, due to the sensitivity limit of fluorescent imaging, it's difficult to determine the time when cells start to respond to the signal, and how variable that is from cell to cell. The posterior cells may be more variable in either spatial or temporal responses compared to the anterior and we are not able to distinguish that. However, signaling dynamics is not necessarily a good function of position or time either, there is no evidence for that in our results here. The 83% correlation is thus striking for the posterior progenitors indicating a certain robust logic in the GRN to capture a strong (even short-lived) response to Shh, regardless of position or time. This is an interesting possibility (we do not claim it a mechanism as we have not tested it with perturbations) that challenges the prevailing view in the field that these progenitors integrate Shh exposure over time, or that they acquire positional information by reading a gradient.

The discussion has been modified to be more nuanced about these points.

Thus, while this paper represents an example of what the community needs to do to gain a better understanding of robust patterning under variability, the provided data is not always sufficient to make clear conclusions regarding the functional consequences of signaling dynamics.

We quite agree. Together with the reviewer, we look forward to seeing the publication of some recent, independent progresses overcoming the challenges in our work by other colleagues.

Reviewer #2 (Public Review):

Summary:

In this work, Xiong and colleagues examine the relationship between the profile of the morphogen Shh and the resulting cell fate decisions in the zebrafish neural tube. For this, the authors combine high-resolution live imaging of an established Shh reporter with reporter lines for the different progenitor types arising in the forming neural tube. One of the key observations in this manuscript is that, while, on average, cells respond to differences in Shh activity to adopt distinct progenitor fates, at the single cell level there is strong heterogeneity between Shh response and fate choices. Further, the authors

showed that this heterogeneity was particularly prominent for the pMN fate, with similar Shh response dynamics to those observed in neighboring LFP progenitors.

Strengths:

It is important to directly correlate Shh activity with the downstream TFs marking distinct progenitor types in vivo and with single cell resolution. This additional analysis is in line with previous observations from these authors, namely in Xiong, 2013. Further, the authors show that cells in different anterior-posterior positions within the neural tube show distinct levels of heterogeneity in their response to Shh, which is a very interesting observation and merits further investigation.

Weaknesses:

This is a convincing work, however, adding a few more analyses and clarifications would, in my view, strengthen the key finding of heterogeneity between Shh response and the resulting cell fate choices.

We thank the reviewer for the comments, due to the age of the work and logistic constraints, we are unable to perform further experiments and analysis to address some of the concerns. We revised conclusions and viewpoints accordingly to reflect reviewer concerns.

Recommendations for the authors:

Reviewer #1 (Recommendations for The Authors):

Minor comments:

y-axis label suddenly changes to Ptch2-reporter level in Figure 5. Is what is plotted different from what is seen as examples in Figure 3?

Thanks! Figure 5 tracks are as Figure 3B, this has been annotated in the figure legends.

There are random bounding boxes in some of the figures.

Sometimes the m in "More dorsal" is stylized with a capital M and sometimes not. It is somewhat confusing as a name for cell types but it is fine if no alternative can be found.

This study unfortunately does not include markers that distinguish the interneurons dorsal to pMNs. We categorized them collectively as "more dorsal".

Response-time is defined as "the amount of time with an above-basal Shh response". This seems to me as the definition of response duration. I would assume that response-time, means the time it takes until a response is first observed. Please consider changing this.

We did not use "duration" because a response time course recorded in these tracks may include multiple durations (on and off). The duration of exposure/response has been specifically used in the field as a single period of response. So it's a sum of active responding time here. Clarified in the text.

Reviewer #2 (Recommendations for The Authors):

(1) The authors address several possible setbacks of transforming the measured fluorescence intensity of the patched reporter into a readout of the Shh signaling activity over time, however, one aspect that isn't directly addressed is the potential effect of differences in the z position of analyzed cells. These could, at least in principle, be sufficient to introduce significant noise in the fluorescence measurements. Can the authors subset their datasets by initial, as well as average, z position and then re-

examine the measured trends for both Shh activity and the intensity of the cell fate reporters used in the study?

The zebrafish early neural plate/tube has a small thickness in z in dorsal-ventral imaging and the tissue is transparent. The depth-associated scattering contributes very little, if at all to the fluorescent signals in the imaged time window. This can be seen in the nuclear/membrane signal of the movies, which is largely uniform across the tissue in z in the neural tissue. It can also be seen that the notochord cells, further ventral, appears to be dimmer.

(2) It is critical for the validity of this study that the intensity of the patched reporter introduced by the authors in 2012, and used again in this study, faithfully represents the signaling activity of Shh. In this study, the authors provide measurements of the transcriptional rate of Kaede and additional modeling for this purpose. However, an important point is to determine how sensitive is the reporter to changes in Shh signaling of different magnitudes?

We consider this BAC reporter line a good (probably still the best live reporter) one as it resolves the endogenous gradient up to the dorsal interneuron domains (Huang et al., 2012, Xiong et al., 2013) and responds well to perturbations (Notch, Cyclopamine, etc). But it's true that we don't have information of how sensitive it responds to changes of different magnitude. As far as we know, there is no in vivo, single cell information of how Shh targets respond to signaling of different magnitudes.

(3) To strengthen the previous point, it would be nice to extend the analysis in Figure 2, at least partially, using other readouts for Shh activity (e.g. GBS-GFP)?

We have used a GBS-RFP line previously and found it to be lower resolution in terms of showing the DV gradient, compared to ptch2:kaede.

(4) It is unclear to me what is the relevant time window during which cells respond to Shh in the anterior versus posterior domains to determine progenitor specification. This is a concern to me, since: i) the average heterogeneity of Shh activity seems to increase strongly in time (Figure 2A/C); and ii) it is important to exclude that the finding of heterogeneous relationship between Shh activity and fate choices is largely driven by later timepoints, where potentially its activity is no longer relevant for cell fate specification. Can this point be clarified when this data is introduced in the manuscript and further discussed?

Yes this is an important point/caveat of live signaling and fate tracking. As discussed in the manuscript, due to the sensitivity limit of fluorescent imaging, it's difficult to determine the time when cells start to respond to the signal, and how variable that is from cell to cell. The posterior cells may be more variable in either spatial or temporal responses compared to the anterior and we are not able to distinguish that.

(i) The ptch2:kaede reporter variability is higher in terms of magnitude (the signal gets brighter) in later times but the heterogeneity (overlap between difference cell fate groups) is lower in later times

(ii) Similarly, the heterogenous relationship is more pronounced in early time points. Since we do not know exactly when the activity becomes no longer relevant (from our earlier studies we do think that the cells become specified early, when Shh signaling is noisy), we modelled the response profile and searched for a good predictor. The maximum response stands out, particularly as a good indicator for the posterior cells, suggests an early window/time of specification.

Discussion has been modified to clarify these points.

(5) Is the response of the patched reporter, as well as cell fate reporters, to defined concentrations of exogenously provided Shh heterogeneous, for instance, in in vitro experiments?

Well-controlled (e.g., microfluidics and labeled Shh molecules) in vitro experiments will be fantastic future directions. Existing tissue explant + Shh dose approaches do not resolve the heterogeneity of exposure at single cell level but may be helpful in testing the limits and variabilities at different magnitudes.

(6) The source of noise in this system is not entirely clear to me. The authors seem to attribute the heterogeneity they observe to the way cells respond to Shh, but can it be excluded that the morphogen profile is itself noisy to start with? It is currently difficult to distinguish between these two possibilities, given that the Shh activity reporter used in this study is itself a transcriptional output of the pathway. Can the distribution of Shh itself be analyzed (even if in immunostainings) during neural tube formation?

Yes we fully agree. More quantitative analysis may help dissecting the sources of noise. The morphogen profile (particularly through time) will be great. Currently no reagent is available to achieve that. Studies using an engineered morphogen or tagged morphogen suggest that the pattern through tissue reasonably captures simple diffusion dynamics. However, at single cell level considerable randomness may still remain and difficult to quantitatively compare with still staining.

(7) It is unclear to me how the authors define the ultimate cell fate of cells in their analysis in Figure 6. The brief description in the methods and in the manuscript seems to suggest that, in combination with marker expression, the cell position is used as a criteria to assign the fate to the progenitors - if this is the case, I guess the observed relationship in Figure 6 with LMDV distance is almost a control? This could be clarified for the readers.

Yes indeed Figure 6 is a control as LMDV distances lead to final positions which form part of our determination of cell fates.

As established by others' and our previous studies (See also Fig.1A), the identification of MFPs and LFPs in zebrafish spinal cord is very robust. The MFPs are the apical constricted single column of cells along the midline on top of the notochord, and the LFPs are the 2 columns of cells next to MFP on both sides. LFPs' expression of *olig2:gfp* did vary more in the posterior (timing of response/commitment could be a factor as the reviewer pointed out), but eventually the cells at those positions will be V3 interneurons or floor plates and have not been observed to make motoneurons. There are 3 low *Olig2:GFP* pMNs in the anterior dataset (Fig.2B') and 3 high *Olig2:GFP* LFPs in the posterior dataset (Fig.2D') that we checked carefully.

The methods of fate determination are described in detail in methods.

(8) The graphs in Figures 6 and 7 are difficult to interpret. What proportion, and absolute number, of cells are "mis specified" when the authors show the distinct colored lines in the pMN, LFP or more dorsal domains? How do the authors determine where each cell fate domain begins and ends to access for "mis-specified" cells? Can the authors also provide the corresponding experimental images in the figure?

We apologize for the difficulties to interpret these figures. The graphs are a ranked list of all cells using the specified metric. The visual is to help generate an intuition of how mixed vs clear-cut the pattern is given the tested metric. They are not to be interpreted as the actual pattern in the tissue and there are no data images that show these patterns.

(9) Given the experimental limitations/technical challenges discussed by the authors during the paper, the score of around 90% of predictability of cell fate choices is rather high in the anterior domain, suggesting a minor functional role for heterogeneity in this region. Even for the posterior domain, the score of 83% predictability based on the maximum response to Shh is still relatively high. In my view, this author's conclusions should be adjusted to make this difference clearer in the abstract and discussion, highlighting that the heterogeneity between Shh response and cell fate choices, particularly in the pMN fate, are stronger in the posterior domain affecting the precision of cell fate decisions particularly in this region. Can the authors further comment on potential mechanisms driving this difference?

Yes – we agree that most cells are actually accurate in such a highly dynamic tissue. In the literature, the view has been more focused on how the GRN enables this accuracy. We therefore highlighted the heterogeneity and limit of accuracy of the GRN here.

We have added the fact that the Shh response is still the main determinant of the pattern despite the heterogeneity in the Discussion. We also further discussed possibilities of the anterior posterior differences.

(10) Following up from the previous point, the data in Figure 7 suggests that there might be different underlying mechanisms in how anterior and posterior cells interpret the Shh profile, with anterior cells potentially responding to the integrated concentration of Shh (since response time, average response, or maximum response to Shh all provide similar predictability scores for cell fate choices). In contrast, only the maximum response to Shh can provide a good prediction of posterior cell fate, consistent with a more instantaneous response to morphogen concentration (and thus potentially more error-prone measurement of the Shh profile?). This is a very interesting observation in my view. Could this be further tested?

Thank you. Yes we found this very interesting too. We discussed the possibilities, including the reviewer's suggestion that these cells may have different contexts or strategy to interpret the signal. It is also possible that the anterior cells use the same strategy (maximum response at an early time) and the subsequent response/duration do not matter to their fate commitment. A precise approach to shut down Shh response dynamics in single cells (e.g., optogenetics) will enable the test of these ideas. We hope following up studies will take such approaches.

<https://doi.org/10.7554/eLife.96980.2.sa0>