Cells change their sensitivity to an EGF morphogen gradient to control EGF-induced gene expression

Jeroen Sebastiaan van Zon¹,², Simone Kienle², Guizela Huelsz-Prince², Michalis Barkoulas³,⁴ & Alexander van Oudenaarden¹,⁵

How cells in developing organisms interpret the quantitative information contained in morphogen gradients is an open question. Here we address this question using a novel integrative approach that combines quantitative measurements of morphogen-induced gene expression at single-mRNA resolution with mathematical modelling of the induction process. We focus on the induction of Notch ligands by the LIN-3/EGF morphogen gradient during vulva induction in Caenorhabditis elegans. We show that LIN-3/EGF-induced Notch ligand expression is highly dynamic, exhibiting an abrupt transition from low to high expression. Similar transitions in Notch ligand expression are observed in two highly divergent wild C. elegans isolates. Mathematical modelling and experiments show that this transition is driven by a dynamic increase in the sensitivity of the induced cells to external LIN-3/EGF. Furthermore, this increase in sensitivity is independent of the presence of LIN-3/EGF. Our integrative approach might be useful to study induction by morphogen gradients in other systems.
How cells in developing embryos interpret external signals to make robust cell fate decisions is still an open question. This is particularly challenging for the induction of spatial cell fate patterns by morphogen gradients, where induced cells do not just respond to the absence or presence of a signal, but rather to its exact local concentration. Understanding how the quantitative information contained in morphogen gradients, coupled with its read-out by the downstream gene regulatory network, generates such dynamics is challenging. Quantitative analysis of the induced gene expression dynamics can provide novel insights into these questions. In particular, quantitative measurements can be used to test and constrain mathematical models of the underlying gene regulatory network. In this way, one can identify in a systematic manner which parameters in the model are essential to explain the observed dynamics. Here, we use such a quantitative approach to study *C. elegans* vulva induction, a classical model of spatial cell fate patterning by a morphogen gradient.

The *C. elegans* vulva is induced from six equivalent vulva precursor cells (VPCs): P3.p-P8.p (Fig. 1a). Guided by a spatial LIN-3/EGF gradient from the anchor cell (AC), each VPC adopts $1^\circ$, $2^\circ$ or $3^\circ$ fate, in a precise spatial pattern: P6.p, the cell closest to the AC, assumes $1^\circ$ fate, the neighbouring VPCs P5.p and P7.p assume $2^\circ$ fate and the remaining VPCs assume $3^\circ$ fate. This cell fate pattern is thought to be established in the following manner: activation of the EGF receptor (EGFR) LET-23 by LIN-3 induces EGF/Ras signalling and subsequent upregulation of Notch ligands *lag-2*, *apx-1* and *dsl-1* in each VPC in a graded manner, depending on the external LIN-3 concentration. Next, the Notch ligands stimulate lateral Notch signalling via the Notch receptor LIN-12, resulting in the inhibition of EGF/Ras signalling in neighbouring VPCs. As a consequence, the higher initial level of Notch ligands in P6.p eventually leads to full inhibition of EGF/Ras signalling in P(5,7).p. In this way, the external LIN-3 gradient is amplified into an all-or-nothing difference in signalling between VPCs, with EGF/Ras signalling and Notch ligand expression restricted to P6.p ($1^\circ$ fate) and high LIN-12/Notch activity in P(5,7).p ($2^\circ$ fate).

The best-studied example of downstream gene expression induced during vulva development is the $1^\circ$ fate marker *egl-17*, a target of the Ras pathway but otherwise not involved in vulva induction. During early induction, a reporter for *egl-17* was induced by LIN-3 in a graded manner, that is, decreasing with

![Figure 1](image_url)
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distance from the AC, in P(5,6,7).p, before its expression was later restricted to P6.2. However, it is currently not clear what Notch ligand expression dynamics is induced by LIN-3, despite the pivotal role of Notch ligands in vulval cell fate patterning. Previous experiments showed expression of reporters for all three Notch ligands in P6.p during vulva induction, but were limited in terms of quantification and time resolution. Specifically, it remains unclear whether Notch ligands show a graded expression pattern during early induction, as was observed for egl-17, or exhibit different dynamics.

To address these questions we systematically quantify Notch ligand expression during vulva induction. We find that Notch ligands are initially expressed at low levels in multiple VPCs but are expressed at high levels and only in P6.p at late induction. Furthermore, we find that this increase in expression level is due to the VPCs increasing their sensitivity to LIN-3/EGF over time and that this change in sensitivity is independent of LIN-3/EGF and Ras signalling.

Results

Dynamic Notch ligand expression during vulva induction. We used single molecule fluorescence in situ hybridization (smFISH)9,10 to visualize and count single mRNA molecules of the Notch ligands lag-2, apx-1 and dsl-1 in individual VPCs in fixed wild-type (N2) animals (Fig. 1b,c). We observed expression in VPCs of lag-2 and apx-1 but not dsl-1 (Supplementary Fig. 1), although there is genetic evidence for a role for dsl-1 in vulva induction.7,11 We observed dsl-1 expression in embryos and also in VPCs when overexpressed from a vulva-specific promoter but not when overexpressed from the dsl-1 promoter (Supplementary Fig. 1). This suggested that dsl-1 is expressed in VPCs at low levels or inaccessible to our smFISH probes and hence we excluded dsl-1 from our analysis.

We tested whether the smFISH probes specifically labelled apx-1 and lag-2 transcripts. First, we induced apx-1 RNAi knockdown by feeding RNAi and observed, by smFISH, significant reduction of expression of apx-1 but not lag-2 (Supplementary Fig. 2a,b). We did not observe a decrease in lag-2 level upon lag-2 RNAi, likely reflecting the variability of RNAi treatment.12 However, the lag-2 expression pattern outside of the VPCs was different from apx-1, showing expression in the AC and distal tip cells (DTCs) (Fig. 1b,c), which is known to express lag-2 (refs 13-15). Finally, expression of both lag-2 and apx-1 was absent from VPCs in most animals in a lin-3(e1417) mutant where lin-3 expression in the AC and induction of vulval cell fate are strongly reduced16 (Supplementary Fig. 2c,d). Together, these results indicated that the lag-2 and apx-1 expression patterns, as observed by smFISH, reflected their induction by LIN-3.

As smFISH requires fixation of the sample, each animal studied provided a snapshot of Notch ligand expression at a particular stage of development. To extract time dynamics from such measurements, we determined the gonad length, defined as the distance between DTCs, using the smFISH measurements. We quantified time dynamics of the gonad length, defined as the number of time points using the expression pattern of lag-2, as observed by smFISH, which reflects their expression level upon induction by LIN-3. Despite the low levels of expression in embryos and in VPCs when overexpressed from a vulva-specific promoter but not when overexpressed from the dsl-1 promoter (Supplementary Fig. 1). This suggested that dsl-1 is expressed in VPCs at low levels or inaccessible to our smFISH probes and hence we excluded dsl-1 from our analysis.

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Conserved expression dynamics in highly divergent isolates. A surprising feature of the Notch ligand expression dynamics was the abrupt increase in expression levels during late vulva induction (Fig. 2a,b). To examine whether this rise in expression is significant for vulva induction, we used smFISH to visualize lag-2 and apx-1 expression in two N2-derived C. elegans isolates, CB4856 and JU775, that are highly divergent from the laboratory strain N2 (ref. 18). For both isolates, we observed a clear transition from low to high Notch ligand expression in P6.p (Fig. 3). Surprisingly, despite small differences, many features of the observed expression dynamics were conserved between N2, CB4856 and JU775. In particular, we observed that in all three cases during early induction apx-1 was expressed more highly than lag-2, whereas after the transition to high Notch ligand expression that situation was reversed. In addition, the timing of Notch ligand expression dynamics, as quantified by gonad length extension, was similar between the three strains. Together, the observed similarities suggest that these features of Notch ligand expression are important for vulva induction.

Expression dynamics is driven by EGF/Ras signalling. Next, we examined whether the increase in Notch ligand expression during vulva induction reflected an integration of the LIN-3 signal, for example, by steady accumulation of long-lived mRNA molecules, or corresponded instead to an instantaneous read-out of the external LIN-3 signal. We inhibited the EGF/Ras signalling pathway using a temperature-sensitive sos-1 mutant, where at 25°C EGF/Ras signalling is inhibited19. We shifted sos-1(ts) animals in the late-induction stage (10-12 h) to 25°C for different time intervals, and measured the lag-2 and apx-1 mRNA level in P6.p at the end of the interval. We found that in the absence of EGF/Ras signalling lag-2 and apx-1 mRNA levels decayed rapidly, with half-lives of ~10 min (Fig. 2e,f). We observed no decay in wild-type animals that underwent the same treatment (Fig. 2e,f and Supplementary Fig. 2e,f). Hence, EGF/Ras signalling dynamics and mRNA turnover are approximately in steady state on the timescale of vulva induction and Notch ligand expression forms a nearly instantaneous read-out of EGF/Ras signalling and the external LIN-3 signal.

During normal development Notch ligand expression is controlled by a combination of the LIN-3 signal and lateral Notch inhibition. Hence, the observed expression dynamics in P6.p could be driven by EGF/Ras signalling in P6.p and/or by (the relief of) inhibitory Notch signalling from P(5,7).p. To decouple these contributions, we measured Notch ligand expression in a Notch receptor mutant, lin-12(fj), where Notch signalling between VPCs is lost20. We choose this approach over vulva-specific lin-12 RNAi, where inhibition of Notch signalling occurs...
with variable efficiency\textsuperscript{21}. However, lack of Notch signalling in \textit{lin-12(lf)} animals typically leads to two ACs\textsuperscript{20}, which likely increases the concentration and range of the LIN-3 gradient. Surprisingly, we found that in \textit{lin-12(lf)} animals Notch ligand expression dynamics in P6.p was very similar to wild type, despite the expected accompanying increase in LIN-3 dosage (Supplementary Fig. 3b,c), an observation addressed further below.

In \textit{lin-12(lf)} animals, \textit{lag-2} and \textit{apx-1} were expressed in multiple VPCs, with high expression levels in P5.p, P6.p and in ~50% of P7.p at the late vulva induction stage (Fig. 4c and Supplementary Fig. 3a), consistent with the cell fate transformations observed in \textit{lin-12(lf)} animals\textsuperscript{22} and likely due to the increased AC number\textsuperscript{21}. We found that Notch ligand expression depended strongly on the distance from the ACs, the source of the LIN-3 gradient, presumably reflecting the shape of the external LIN-3 gradient. To quantify this effect, we plotted the \textit{lag-2} and \textit{apx-1} mRNA levels in each VPC versus its distance along the anteroposterior axis to the centre of the two ACs, which cluster closely together in the gonad (Fig. 4a,b,d,e). We found that \textit{lag-2} and \textit{apx-1} expression levels decreased monotonically with distance from the ACs, with an ~15–20 $\mu$m half-width (Fig. 4d,e,i). We observed that the expression dynamics of \textit{lag-2} and \textit{apx-1} shared characteristics with wild-type (Fig. 2a,b) and \textit{lin-12(lf)} animals (Fig. 4a,b,d,e and Supplementary Fig. 3a–c): in both cases, \textit{apx-1} was expressed earlier and more widely than \textit{lag-2} during the early-induction stage (0–4 h), whereas \textit{lag-2} and \textit{apx-1} expression levels increased markedly in the late stage (10–12 h), with \textit{lag-2} expression levels rising above \textit{apx-1} levels. Hence, these aspects of Notch ligand expression were controlled exclusively by EGF/Ras signalling.

Mathematical modelling of expression dynamics. We then used mathematical modelling to systematically examine how the observed transition in Notch ligand expression is regulated by EGF/Ras signalling. Previous models focused on crosstalk between the EGF/Ras and Notch pathways in cell fate assignment\textsuperscript{23–25}. Here, we focus purely on Notch ligand induction by the LIN-3 gradient and EGF/Ras pathway, without Notch signalling. The signalling network underlying vulva induction, as deduced from genetics experiments, is highly complex\textsuperscript{6,26}. Explicitly modelling this full complexity would yield a model whose parameters we cannot constrain with our experimental data. Instead, we searched for the simplest model that could reproduce the experimental data for wild-type animals and key mutants. For this, we used the following approach: first, we identified simple candidate models, based on the current knowledge of EGF/Ras signalling\textsuperscript{26–28} and Notch ligand induction\textsuperscript{13}, that reproduced the transition in Notch ligand expression in wild-type animals. Then, by comparing the predictions of the different candidate models for key mutants
we systematically eliminated all but a single model, strongly constraining the possible mechanisms underlying the transition.

We based our models on the following observations: LIN-1 represses lag-2 expression in VPCs and inhibition of the repressive action of LIN-1 by EGF/Ras signalling is responsible for inducing lag-2 in P6.p. However, there is evidence that LIN-1 can also activate gene expression. Finally, on inhibition of LIN-1 repression, lag-2 expression is induced by one or more unknown activators. This led us to consider three different models of increasing complexity. In the simplest model (Model A, Fig. 5a), EGF/Ras signalling stimulates the transition of LIN-1 from its unphosphorylated repressive form (LIN-1) to its phosphorylated inactive form (LIN-1-P), after which expression of lag-2 is induced by the activator A. In this model, three fundamentally different mechanisms could underlie the transition in Notch ligand expression: a change in LIN-3 level during induction (Model A1); a change in sensitivity of the EGF/Ras pathway to LIN-3 (Model A2); or a change in the amount or activity of the activator A (Model A3). The next model (Model B, Fig. 5a) is similar to Model A, but here EGF/Ras signalling stimulates a transition in LIN-1 from a repressive form (LIN-1) to an activating form (LIN-1-P). We assumed that induction of lag-2 expression occurs at low rate when either LIN-1-P or the activator A are bound to the lag-2 promoter individually and at a high rate when bound simultaneously. As for Model A, here the transition can be driven by a change in the LIN-3 level (Model B1), in the sensitivity of the EGF/Ras pathway to LIN-3 (Model B2) or in the level of the activator A (Model B3). Finally, we considered a model (Model C, Fig. 5a) where EGF/Ras signalling not only controls the transition between LIN-1 and LIN-1-P but also the amount of activator A. In this case, the transition in Notch ligand expression can be driven by a change in external LIN-3 (Model C1) or in the sensitivity of the Ras pathway to LIN-3 (Model C2).

To calculate expression levels from the models in Fig. 5a, we use the following approach: we assumed that LIN-3 forms a concentration gradient outside the VPCs with its maximum at P6.p. LIN-3 activates the EGFR LET-23 on the VPC surface. The level of EGF/Ras activation in each VPC depends on the total amount of activated EGFR at the VPC surface. The phosphorylation rate of LIN-1 (all models) and the activation rate of the activator A (Model C) depend on the EGF/Ras signalling level. To connect the levels of LIN-1, LIN-1-P and A to lag-2 expression, we assumed that the lag-2 transcription rate depends in a model-specific manner on the probability of LIN-1, LIN-1-P and A bound to the lag-2 promoter (Supplementary Fig. 8). Such 'thermodynamic models' of gene expression can successfully reproduce experimental observations. As a result, we obtained expressions for the lag-2 transcription rate that depend on five (Model A) or seven parameters (Models B and C). For the full expressions and more details, see the Methods section. Finally, we assumed that all the above processes were in steady state on the timescale of induction and, hence, the models were not explicitly time-dependent. Instead, the expression dynamics in Figs 2–5 were due to changes in time of the model parameters.

Dynamics is driven by a change in sensitivity to LIN-3. Notch ligand expression in P6.p appears unaffected by Notch inhibition from P(5,7).p (Supplementary Fig. 3b,c). Therefore, we used the above models, without Notch signalling, to model Notch ligand expression in P6.p in wild-type animals. We tested the ability of all models to reproduce the wild-type data for lag-2 expression, which showed the strongest transition in expression level. We fitted all models to the lag-2 expression levels in P6.p for early (1–4h) and late (9–12h) induction and each model could correctly reproduce the transition (Fig. 5b and Supplementary Fig. 5). These fits were not unique: many combinations of parameter values reproduced the observed transition.

Next, we tested whether the increase in Notch ligand expression reflected an increase in external LIN-3. The corresponding models (A1, B1 and C1) were very sensitive to changes in LIN-3 level: in general, they predicted significant rises

Figure 3 | Notch ligand expression dynamics during vulva induction in wild C. elegans isolates. (a) lag-2 and (b) apx-1 mRNA levels in P5.p, P6.p and P7.p as a function of time in the wild C. elegans isolate CB4856 (n = 32 animals). Shown in comparison are mRNA levels in P6.p in N2 animals (grey markers). The magenta and cyan markers indicate the mRNA levels in P6.p in the animals in panel (c). To facilitate comparison with the data in Fig. 2, we used the expression in Fig. 1e for N2 animals to convert gonad length into time. (c) Visualization of single-mRNA molecules of lag-2 (red) and apx-1 (green) in P6.p in CB4856 animals at the early (top) and late (bottom) vulva induction stage. Scale bar, 3 μm. (d) lag-2 and (e) apx-1 mRNA levels for the wild C. elegans isolate JU775 (n = 44 animals). (f) Visualization of lag-2 (red) and apx-1 (green) mRNA molecules in P6.p in JU775 animals.
in expression level on increasing LIN-3 dosage. We experimentally increased LIN-3 dosage using a lin-3(++) mutant that carried an integrated transgenic array, syIs1, that strongly overexpresses lin-3 in the ACs. Surprisingly, in lin-3(++) mutants, the lag-2 expression level in P6.p did not increase, for both early and late induction (Fig. 5c). However, the LIN-3 dosage in this mutant had clearly increased, as we observed a strong increase in lag-2 expression in all other VPCs (Supplementary Fig. 3b-d). This result was consistent with the observed lack of change in expression in the lin-10(0) mutants that contained an extra AC. All models, even A1, B1 and C1, could reproduce this lack of increased expression at the late stage, if the LIN-3 level at that stage was so high that all EGFRs were saturated by bound LIN-3 and hence further increases in LIN-3 had little effect. However, model A1 could not reproduce the lack of change in expression at the early time point and hence we considered this model inconsistent with the experimental data. We excluded the other LIN-3-dependent models, B1 and C1, for similar reasons (Supplementary Fig. 5). We found the same transition in lag-2 expression in a let-60/Ras gain of function mutant, where Ras signalling is constitutively active in all VPCs (Supplementary Fig. 4e-g). Together, these results showed that the transition in Notch ligand expression was not due to an increase of LIN-3 level or LET-23 activation.

Next, we tested whether the transition in expression level depended on LIN-1. We quantified lag-2 expression in a mutant, lin-1(0), where vulval cell fate is induced in almost all VPCs, even in animals without an AC. If the change in expression is driven by a change in Ras signalling that exclusively had an impact on LIN-1 and LIN-1-P levels (Model A2 and B2), the lag-2 expression level at the early- and late-induction time points was predicted to be identical (Fig. 5d). However, if the change in expression is due to an increase in the amount of the activator A either independent (Model A3 and B3) of or dependent on Ras signalling (Model C2), the transition from low to high lag-2 expression was predicted to occur even without LIN-1 (Fig. 5d). Indeed, in lin-1(0) animals we observed a strong rise in lag-2 expression level in all VPCs, with few differences between VPCs (Fig. 5d, Supplementary Fig. 4a–d). This ruled out Models A2 and B2. Unexpectedly, in lin-1(0) animals lag-2 expression levels never reached the fully induced levels observed for wild-type induction and were overall lower than in wild-type animals. Previous experiments showed that the main role of LIN-1 is to inhibit vulval cell fate and lag-2 expression, which is supported by our observation of lag-2 expression in all VPCs in lin-1(0) animals. However, the assumption that LIN-1 only acts as a repressor (Model A3) predicted that absence of LIN-1 would lead to full induction of lag-2 (Fig. 5d). However, if LIN-1-P...
Figure 5 | Mathematical modelling of induction of Notch ligand expression. (a) Overview of the different models of the EGF/Ras pathway considered in explaining the transition in lag-2 expression, taking into account the ligand LIN-3, receptor LET-23, the transcription factor LIN-1 and activator A. In model B, but not Model A, LIN-1-P activates lag-2 expression. In Model C, but not Models A and B, LIN-1 and the activator A are both controlled by Ras signalling. In the models, the change in lag-2 expression is controlled either by a change in LIN-3 level (Models A1-C1), a change in sensitivity of the Ras pathway to LIN-3 (Models A2-C2) or a change in the amount of activator A (Models A3-B3). As Models A1-C1 are similar, here only Model A1 is considered. See Supplementary Fig. 5 for results on all models. (b) Early (1–4 h) and late (9–12 h) lag-2 expression levels in wild-type animals (grey), with n ≥ 24 animals for each time point. Error bars here and in panels (c–d) and (g) indicate s.e.m. Coloured bars show best fits to the wild-type data for Models A1-C2. All models are able to reproduce the data (green tick marks). (c) Early and late lag-2 expression levels in the lin-3(++) mutant for the experiment (grey bars, n ≥ 8 animals) and best fits of Models A1-C2 (coloured bars). Model A1 failed to fit the experimental data (red cross). (d) Same as (e) but in the lin-1(0) mutant (n ≥ 22 animals). Only models B3 and C2 could reproduce the experiment. (e) Visualization of lag-2 (green) and lin-3 (red) mRNA molecules in P6.p and the AC at early (upper panel) and late (lower panel) vulva induction in the lin-1(0) mutant, showing lag-2 and lin-3 expression in the AC. Scale bar, 3 μm. (f) Same as (e) but in the lin-1(0);lin-3(e1417) mutant. A transition in lag-2 expression is visible without lin-3 expression in the AC. (g) Early and late lag-2 expression in the lin-1(0);lin-3(e1417) mutant for the experiment (n ≥ 7 animals) and best fit for Models B3 and C2. Only Model B3 was consistent with the experimental data.

functioned as an activator of lag-2 expression (Models B3 and C2), we could fit the transition with reduced lag-2 expression levels. These results provide the most direct evidence so far for an activating role of LIN-1 in lag-2 expression, perhaps to fine-tune the expression levels.

Finally, we tested whether the change in expression was due to a change in activation that is dependent on the LIN-3 signal and downstream Ras signalling (Model C) or independent of LIN-3 (model B3). We measured lag-2 expression dynamics in a lin-1(0);lin-3(e1417) mutant. Model C2 predicted strongly reduced lag-2 expression in the lin-1(0);lin-3(e1417) mutant due to the lack of LIN-3 input, similar to our observations in the lin-3(e1417) mutant (Supplementary Fig. 2c,d). However, Model B3 predicted that as the change in the amount of activator A occurred independently of the LIN-3 level and Ras signalling, the transition in lag-2 expression level would remain unchanged compared with the lin-1(0) mutant. Indeed, we found that lag-2 expression in the lin-1(0);lin-3(e1417) still showed a clear transition, with the exact levels very similar to what we observed in the lin-1(0) animal (Fig. 5g), while the level of lin-3 in the AC was indeed strongly reduced compared with lin-1(0) animals (Fig. 5e,f). Hence, the transition in lag-2 expression is regulated downstream of LIN-1 in a LIN-3-independent manner, presumably by temporal modulation of the unknown activator(s) of lag-2 expression.

Different threshold for lag-2 and apx-1 expression. We found only model B3 consistent with the key mutant data for lag-2 expression. We then examined whether this model could also
explain the full lag-2 and apx-1 spatial expression profiles in lin-12(0f) animals (Fig. 4). The apx-1 promoter contains sequences similar to the promoter elements, including LIN-1 binding sites, that control lag-2 expression in P6.p\textsuperscript{9}. Hence, we assumed that apx-1 is induced as lag-2, but with different values for the binding rates of LIN-1, LIN-1-P and the activator A to the apx-1 promoter. Assuming that lag-2 and apx-1 expression are both controlled by the same combination of repressive (LIN-1) and inductive signals (LIN-1-P and A), the extended model B3 predicted that in each VPC the mRNA levels \(L_{\text{apx-1}}\) and \(L_{\text{lag-2}}\) obeyed the relation:

\[
L_{\text{apx-1}} = \frac{L_{\text{max}}^{\text{apx-1}} \kappa L_{\text{lag-2}}}{L_{\text{lag-2}} + (\kappa - 1)L_{\text{lag-2}}^2},
\]

which depends only on the two variables \(L_{\text{lag-2}}^{\text{max}}\), the maximally induced mRNA level for ligand j, and a parameter \(\kappa\), the ratio between the thresholds for induction of lag-2 and apx-1 by the activator A. Here \(\kappa > 1\) would mean that apx-1 has a lower threshold to induction by Ras signalling than lag-2. (Supplementary Note 8). In agreement with this prediction, we found that for both wild-type and lin-12(0f) animals (Fig. 6) the lag-2 and apx-1 mRNA levels for all VPCs largely followed the predicted curve. We then used this single-cell data to constrain the parameter \(\kappa\). First, we measured \(L_{\text{lag-2}}^{\text{max}}\) by averaging mRNA numbers for P6.p in wild-type and lin-12(0f) animals at \(>9\) h, yielding \(89 \pm 3\) transcripts for lag-2 and \(53 \pm 2\) transcripts for apx-1 (Supplementary Fig. 3e,f), similar to the average mRNA levels in the lin-3(++) overexpression mutant (Fig. 5c and Supplementary Fig. 3e,f). We then fitted equation (1) to the experimentally observed correlation data by varying \(\kappa\) (Fig. 6 and Supplementary Fig. 6), observing good agreement for \(\kappa \approx 5 - 10\), that is, apx-1 having a 5- to 10-fold lower threshold to induction by EGF/Ras signalling compared with lag-2.

Using the measured values of \(L_{\text{lag-2}}^{\text{max}}\) and \(\kappa\), we were able to find many fits that reproduced both the time dynamics and spatial expression profiles of both lag-2 and apx-1 (Fig. 4a,b,f-i) as well as the gene expression dynamics for the lin-3(++) and lin-1(0) mutants, with parameters constrained to a small region of parameter space (Supplementary Fig. 7). See the Methods section for details of the fitting procedure. All these fits showed the same increase in activator A over the course of induction while the LIN-3 gradient decay length remained approximately constant. In the model, the difference in threshold is sufficient to explain that during the early stage apx-1 is expressed earlier and more widely than lag-2 (0–4 h, Fig. 2a–d), even though both are induced by the same signalling pathway.

**Discussion**

It is becoming increasingly clear that morphogen gradients can induce complex temporal programmes of gene expression\textsuperscript{2}. We quantified gene expression induced by the LIN-3 gradient during vulva induction and found that Notch ligand expression was highly dynamic, with clear differences between lag-2 and apx-1 both in timing and spatial extent. In particular, we found that during early vulva induction apx-1 expression is induced in multiple VPCs, even in P4.p and P8.p, which are far away from the AC, providing the most unambiguous evidence to date that LIN-3 acts as a long-range signal. Subsequently, expression of lag-2 and apx-1 became restricted to P6.p, the VPC assuming 1° fate. After this, we observed a striking transition from low to high Notch ligand expression in P6.p. We observed similar dynamics in a mutant lacking lateral Notch signalling, indicating that the relative time and spatial extent of Notch ligand induction as well as the transition in their expression level are regulated by EGF/Ras signalling alone. Finally, we found that the transition in expression level in P6.p was robust to large changes in LIN-3 dosage.

Our experiments provide no conclusive function for the transition in Notch ligand expression in P6.p. However, we do find similar expression dynamics in two divergent wild C. elegans isolates, suggesting a significant role for this transition in vulva induction. An intriguing observation is that the rise in Notch ligand expression occurs after lag-1 and apx-1 expression has been restricted to a single VPC, a process that is often thought to be the main consequence of Notch signalling during vulva induction. However, Notch signalling acts twice to regulate cell fate decisions during vulva induction: Before the VPC S phase lin-12 influences a 1° versus non-1° fate decision in P6.p and after S phase induces 2° fate decision in P(5,7).p\textsuperscript{37}. The observed timing of the transition in Notch ligand expression suggests that the first decision might occur when Notch ligand expression is low and that the expression levels rise in time for the later decision.

By fitting mathematical models to the experimental results, we systematically tested different potential network topologies of the EGF/Ras signalling network. For each network topology we examined different mechanisms for the observed transition in Notch ligand expression levels. We found that only a single model (Model B3) was consistent with all our experimental observations in different mutants of the EGF/Ras pathway. In this model, the transition in Notch ligand expression is not due to changes in level of the external LIN-3 signal, but instead due to an intrinsic, LIN-3-independent change in sensitivity to external LIN-3. We extended this model to fit expression data for both lag-2 and apx-1 by only assuming different rates of binding of the Ras effector.

**Figure 6 | Single-cell correlation in Notch ligand expression.** (a, b) Single-cell correlation between apx-1 and lag-2 mRNA level in P(5–7).p in (a) wild-type animals (\(n = 73\)) and P(4–8).p in (b) lin-12(0f) animals (\(n = 111\)). Line indicates a fit to equation (1) with the value of \(\kappa\) as shown in the figure. The values between brackets give the 95% confidence interval for \(\kappa\), as obtained by bootstrapping. The white marker indicates the observed average maximally induced levels (\(L_{\text{apx-1}}^{\text{max}}, L_{\text{lag-2}}^{\text{max}}\)). Owing to the absence of lateral Notch inhibition in lin-12(0f) animals, multiple VPCs show high levels of Notch ligand expression.
LIN-1 and the activator A to their binding sites in the lag-2 and apx-1 promoters. Previously, it was found that induction of lag-2 expression by EGF/Ras signalling was controlled by promoter elements that are also present in the apx-1 promoter3. Our results suggest that the observed differences between lag-2 and apx-1 expression could be due to small changes in affinity of transcription factors to these promoter elements3.

The change in intrinsic sensitivity underlying the transition in Notch ligand expression could have occurred at several levels of the EGF/Ras pathway. For instance, in the model the sensitivity is determined by the level of the EGFR LET-23, the transduction of EGFR activity by EGF/Ras signalling and the threshold of Notch ligand expression to activation by EGF/Ras signalling (Supplementary Notes 1–3). It is known that expression of the EGFR between increases in P6.6 during induction27, which would have been a natural mechanism to generate the change in sensitivity to the LIN-3 signal. It is therefore surprising that we still observe a clear transition in Notch ligand expression level in lin-1(0);lin-3(e1417) animals, clearly indicating that this transition is independent of the presence of the LIN-3 signal and the EGF/Ras signalling pathway.

Previous experiments have shown that lag-2 expression not only requires inhibition of the repressive action of LIN-1 by EGF/Ras signalling, but also the action of one or more unknown activators present in all VPCs13. Our results show that temporal regulation of these activators might explain the change in sensitivity we observed in the lin-1(0);lin-3(e1417) mutant. Our model makes a strong prediction of the expression dynamics on loss of the activator (Supplementary Fig. 5): in this case, Notch ligand expression remains at a low constant level during the entire induction process, reflecting basal induction by the activator LIN-1-P. Similarly, the model predicts that loss of the temporal regulators should result in low, constant Notch ligand expression during induction. In general, the observed dynamic changes in sensitivity point to a so far unrecognized temporal aspect of the response to the external LIN-3 signal. It would be interesting to examine whether the change in sensitivity to LIN-3 of Notch ligand induction is part of a larger temporal programme. For instance, it might be possible that other genes expressed in 1° fate cells, including other Ras targets, might show concomitant changes in expression.

In conclusion, our study showed that during vulva induction downstream gene expression is not controlled exclusively by the external LIN-3 gradient and lateral Notch inhibition but also by the intrinsic modulation of the downstream signal by the receiving cells. To obtain these results we relied crucially on smFISH to quantify with high precision differences in expression levels both between different cells and in time. In addition, even though smFISH requires fixation of the animals, we could still obtain dynamical information by using anatomical markers, in our case gonad length, or by precisely timed perturbations to the signalling network. Finally, we analysed the smFISH data using mathematical models of the signalling network, which proved essential in comparing the different potential mechanisms underlying the observed dynamics. Together, these results show that the combination of quantitative smFISH data and mathematical modelling can be a powerful tool to dissect the dynamics of signalling pathways in development.

Methods

C. elegans strains and culture. All strains were handled according to the standard protocol28. Wild-type nematodes were strain N2. The following mutations and integrated transgenic arrays were used in this study: LGII: rrf-3(pk1426)39, LGII: lin-12(e941)34, LGIV: lin-1(n304)34, lin-3(e1417)32, sor-1(cs28)32, LGV: sos-1(n941)35, al-56 [lag-2p::gfp unc-119(+); C176], LGX: syd-1[lin-3(+); unc-1]31, 34. In addition, we used the following extrachromosomal transgenic arrays: lin-31::dsl-1, dsl-1p::dsl-1. To create the lin-3(n304)lin-3(e1417) double mutant, which is phenotypically similar to the lin-(n304) mutant, we followed the approach in ref. 42. Specifically, we picked lin-3(e1417) homozygous animals from the progeny of double mutant heterozygous hermaphrodites by selecting those that showed the Vul variety (Vul) phenotype. These animals could be either lin-1(n304) heterozygous or lin-1(+); lin-3(n304) homozygous, but not lin-1(n304) homozygous, as these would show a multivulva (Muv) phenotype. In the next generation, we obtained the double mutant by selecting animals with a Muv phenotype indicating lin-1 homozygosity. In addition, we confirmed by smFISH that in lin-(n304)lin-3(e1417) animals lin-3 was not expressed in the AC (Fig. 5e,f). All strains were grown at 20°C. To study the effect of the inhibition of EGF/Ras signalling, sos-1(e941) animals were grown on NGM plates at 20°C and shifted to 25°C by moving the plates to a water bath for different periods of time, after which animals were fixed immediately.

To create the lin-31::dsl-1 strain (UW2078), a lin-31::dsl-1–5:unc-54 construct was injected in N2 at a concentration of 80 ng µl−1 in the injection mix. The lin-31::dsl-1–5:unc-54 construct was built by cloning the dsl-1 coding sequence into pB253 (1) as a BglII/NotI fragment using primers dsl-1BglIIF (5’-cgcaacactgtcgaatctttatcctgtc-3’) and dsl-1NotIR (5’-gctggccggccggcgcctcaggggaagagcgt-3’). The dsl-1p–dsl-1 strain was created by injecting an 8.9-kb PCR product in N2 at a concentration of 30 ng µl−1 in the injection mix. This fragment was amplified using primers dsl-1F2 (5’-ctgcggccgagagggccgccc-3’) and dsl-1R2 (5’-agctagcggacctgcatgata-3’) and coupled to Cy5 (GE Amersham) or Alexa594 (Invitrogen). The sequences of the oligonucleotide probes used in this study are listed in Supplementary Data 1. Animals were collected by washing plates with M9 and were fixed in 4% formaldehyde in 1× PBS for 45 min at room temperature. Fixed animals were permeabilized in 70% ethanol overnight at 4°C. Subsequently, animals were incubated with the smFISH probes overnight at 30°C in hybridization solution containing 10% formamide. The next day, animals were washed twice with 10% formamide and 2× SSC, each time followed by an incubation for 30 min at 30°C. To visualize cell nuclei, DAPI was added at 5 µg ml−1 at the last wash step. Microscopy images were acquired with a Nikon Ti-E inverted fluorescence microscope, equipped with a 100× plan-apochromat oil-immersion objective and a Princeton Instruments Pixis 1024 CCD camera controlled by MetaMorph software (Molecular Devices, Downington, PA, USA). Exact three-dimensional positions of smFISH fluorescent spots in each animal were detected using a custom MATLAB (The Mathworks) script, based on a previously published algorithm30. In brief, we first convolved smFISH microscopy images with a Gaussian filter to increase the brightness of spots of the correct size and suppress the background signal. Next, we select candidate spots by thresholding, using a manually determined threshold. We further refined the candidate spots by finding regional intensity maxima within each candidate spot, to separate smFISH spots whose fluorescence signals are partially overlapping. Finally, the resulting smFISH spots were manually assigned to individual VPCs.

RNAi by feeding. Bacteria were grown overnight, concentrated by centrifugation and then seeded onto LB plates containing 1 µM IPTG and 50 µg ml−1 Ampicillin. We performed apx-1 RNAi in a sensitized rrf-3 background34, as observed no knockdown in wild-type animals. Eggs of rrf-3 mutant were collected by bleaching and transferred to fresh RNAi plates. Hatched animals were fixed after ~30 h for smFISH staining. The apx-1 RNAi feeding done was constructed by amplifying a fragment corresponding to apx-1 from N2 DNA using primers apx-1F1 (5’-cacccttcttctgtcataa-3’) and apx-1R1 (5’-ttttcaccaccctagcaccnnaa-3’). This fragment was first cloned into pDONR 221 (Invitrogen) and then transferred to a Gateway compatible L4440 plasmid.

Timelapse imaging. Individual animals were staged on an agar pad with a small patch of E. coli as a food source35. In brief, a single L1 animal was transferred to a small drop of M9 on a 5% agar pad. A cover slip was coated with a small amount of OP50 bacteria transferred from a petri dish with a worm pick and then gently placed on top of the agar pad. To prevent the sample from drying out, the coverslip was sealed with high V. Full-length mRNA of the downstream gene of interest was transcribed in vitro with T7 RNA polymerase. Staged animals were grown at 20°C and briefly imaged at ~1 h intervals at room temperature, using a Nikon Ti-E inverted fluorescence microscope, equipped with a plan-fluor × 40 objective and a Photometrics HQ2 CoolSnap camera, controlled by µManager software (http://www.micro-manager.org).

Fit of gonad length extension. For each frame, we manually measured the gonad length as the distance along the anteroposterior axis between the two DTCs. Entry into and exit from the L1 lethargus was monitored by the reduction of movement and coupled to Cy5 (GE Amersham) or Alexa594 (Invitrogen). The sequences of the oligonucleotide probes used in this study are listed in Supplementary Data 1. Animals were collected by washing plates with M9 and were fixed in 4% formaldehyde in 1× PBS for 45 min at room temperature. Fixed animals were permeabilized in 70% ethanol overnight at 4°C. Subsequently, animals were incubated with the smFISH probes overnight at 30°C in hybridization solution containing 10% formamide. The next day, animals were washed twice with 10% formamide and 2× SSC, each time followed by an incubation for 30 min at 30°C. To visualize cell nuclei, DAPI was added at 5 µg ml−1 at the last wash step. Microscopy images were acquired with a Nikon Ti-E inverted fluorescence microscope, equipped with a 100× plan-apochromat oil-immersion objective and a Princeton Instruments Pixis 1024 CCD camera controlled by MetaMorph software (Molecular Devices, Downington, PA, USA). Exact three-dimensional positions of smFISH fluorescent spots in each animal were detected using a custom MATLAB (The Mathworks) script, based on a previously published algorithm30. In brief, we first convolved smFISH microscopy images with a Gaussian filter to increase the brightness of spots of the correct size and suppress the background signal. Next, we select candidate spots by thresholding, using a manually determined threshold. We further refined the candidate spots by finding regional intensity maxima within each candidate spot, to separate smFISH spots whose fluorescence signals are partially overlapping. Finally, the resulting smFISH spots were manually assigned to individual VPCs.
G(t) = a + bt during the L1 larval stage and G(t) = a + bt + ct² during the L2 larval stage, where t = 0 corresponded to the end of L1 and we constrained the parameters a and b to have identical values in L1 and L2. We defined G(t) as the largest observed gonad length for an animal in lethargus. For three animals we started the experiment after the L1 lethargus and hence we did not know the time relative to the start of L2 at the beginning of the experiment. As a result, we had six fit parameters: the three coefficients a, b and c and three time intervals Δt, measuring the time between the start of the experiment for that animal and the start of L2. We then obtained values for a, b, c and Δt by minimizing the sum of squares error (SSE) of G(t) with respect to the measured gonad length extension.

**Mathematical models of Notch ligand induction.** Following the approach outlined in the main text, we arrived at the following expressions of the lag-2 transcription rate r for the different networks in Fig. 5a. For Model A:

$$r = \frac{x}{1 + t + \frac{1}{t^2}} + \frac{1}{1 + t + \frac{1}{t^2}}$$

For Model B:

$$r = \frac{2x}{1 + t + \frac{1}{t^2}} + \frac{2x}{1 + t + \frac{1}{t^2}}$$

For Model C:

$$r = \frac{2x}{1 + t + \frac{1}{t^2}} + \frac{2x}{1 + t + \frac{1}{t^2}}$$

where 2β is the strength of binding of LIN-1 to the promoter, 2β is the strength of binding of LIN-1-P and x is the strength of binding of the activator A. The parameter φ < 1 indicates for Models B and C the rate of transcription for LIN-1-P and A bound separately to the promoter, compared with the rate for LIN-1-P and A bound simultaneously, which is set to 1. The parameter s represents the strength of Ras signalling and, for a VPC at a distance x to the AC, is given by:

$$s = \beta \sqrt{\frac{n-1}{n}} \frac{\theta}{\theta^2 \lambda^2 + 1 + \frac{\theta}{\theta^2 \lambda^2}}$$

where θ indicates how strongly Ras signalling is induced by a given LIN-3 input, θ indicates the external LIN-3 level, λ is the LIN-3 gradient decay length, p(θ, λ) = θ exp(−θ) and LC is the length of the VPC body along the anteroposterior axis. For a derivation of the above expressions, see Supplementary Notes 1-3. For Models A1, B1 and C1, θ is the only parameter that changes in time, whereas the other parameters remain constant. For models A2, B2 and C2, only β changes as a function of time, while for models A3 and B3 only x changes as a function of time. The models were fitted to the data for the mutants in Fig. 5 as follows: during the fitting procedure we constrained parameter values so that each bootstrap sample. The 95% confidence levels reported in Fig. 6 are for 250 combinations of parameter values, we calculated the change in expression in the experiment after the L1 lethargus and hence we did not know the time relative to the measured gonad length extension.

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