ARTICLES

Variability in gene expression underlies incomplete penetrance

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The phenotypic differences between individual organisms can often be ascribed to underlying genetic and environmental variation. However, even genetically identical organisms in homogeneous environments vary, indicating that randomness in developmental processes such as gene expression may also generate diversity. To examine the consequences of gene expression variability in multicellular organisms, we studied intestinal specification in the nematode *Caenorhabditis elegans* in which wild-type cell fate is invariant and controlled by a small transcriptional network. Mutations in elements of this network can have indeterminate effects: some mutant embryos fail to develop intestinal cells, whereas others produce intestinal precursors. By counting transcripts of the genes in this network in individual embryos, we show that the expression of an otherwise redundant gene becomes highly variable in the mutants and that this variation is subjected to a threshold, producing an ON/OFF expression pattern of the master regulatory gene of intestinal differentiation. Our results demonstrate that mutations in developmental networks can expose otherwise buffered stochastic variability in gene expression, leading to pronounced phenotypic variation.

In 1925 Timoféeff-Ressovsky and Romaschoff independently noticed that individuals harbouring identical mutant alleles often show either mutant or wild-type phenotypes, a property known as incomplete penetrance[1–5]. Such variation can sometimes be traced to differences in genetic background or environmental conditions, but can also arise from random fluctuations in processes such as gene expression[6]. In clonal populations of microbes, these stochastic effects in gene expression can be used as a mechanism for generating phenotypic variation[7–10], and multicellular organisms can also use variability to generate different cell types[11–13]. In general, though, the gene expression patterns of different cells during metazoan development must be coordinated to ensure proper tissue formation. This suggests that stochastic fluctuations in gene expression may be controlled or their effects may be buffered under normal conditions. Here we examined the consequences of random variability in gene expression during intestinal specification by measuring expression in individual *C. elegans* embryos using a fluorescence *in situ* hybridization (FISH) technique capable of detecting single messenger RNA molecules[14]. We found that expression in the wild-type network was highly regular, but that mutations to components of this network that are incompletely penetrant for loss of intestinal cells led to large variations in the expression of a downstream gene. These variations were subsequently thresholded to yield alternative cell fates, showing that incomplete penetrance can result from stochastic fluctuations in gene expression when mutations compromise mechanisms that normally buffer such variability.

Incomplete penetrance in intestinal development

The *C. elegans* intestine consists of 20 cells descended entirely from the E cell, which arises early in embryonic development (Fig. 1a). Intestinal cell-fate specification results from the activity of a short transcriptional cascade, beginning with the maternal deposition of *skn-1* transcripts and ending with the expression of *elt-2*, which then activates hundreds of other genes during intestinal differentiation[15–17] (Fig. 1b). Genetic analyses have shown that *skn-1* activates the genes *med-1* and *med-2* (which are essentially identical)[18,19], as well as *end-3* and *end-1* (ref. 19). END-3 activates *end-1* (ref. 19), and either gene product can activate *elt-2* (refs 20–22), which maintains its own expression through a positive feedback loop[15,16,23]. We examined whether the developmental consequences of mutations to *skn-1* resulted from variability in the expression of genes in this pathway.

We counted the number of mRNAs transcribed from these genes in individual embryos by using a version of FISH that renders each mRNA visible as a single diffraction-limited fluorescent spot[14] (Fig. 1d–f). Co-staining with 4',6-diamidino-2-phenylindole (DAPI) allowed us to count the number of nuclei in each embryo, enabling stage-specific measurement of gene expression (Fig. 1c). We observed that all wild-type embryos beyond the 65-cell stage (when there are 4 cells in the E lineage) contained large numbers of *elt-2* transcripts (Fig. 1g). *skn-1* mutant embryos, however, die in late embryogenesis with most, but not all, embryos lacking intestinal cells[24]. *elt-2* expression in *skn-1* mutant embryos was bimodal, with *elt-2* exhibiting an ON/OFF expression pattern[15] (Fig. 1h).

Intestinal network gene expression dynamics

To study the sources of this variability, we counted transcripts and nuclei in hundreds of differently staged mutant and wild-type embryos, thereby reconstructing the dynamics of the intestinal specification network (Fig. 2a and Supplementary Fig. 1). In the wild type, *med-1* and *med-2* were the first zygotically expressed genes, followed by *end-3*, then *end-1*, and then *elt-2*, in concordance with previous studies[25,26]. We found no evidence for maternal *med-1/med-2* transcripts[19,27], but small numbers of *end-3* and *end-1* transcripts were uniformly distributed throughout one-cell and two-cell embryos, indicating that these originated in the mother's gonad[28] (Supplementary Fig. 2). During periods of peak expression, transcript levels were similar for all these genes and did not display high variability.

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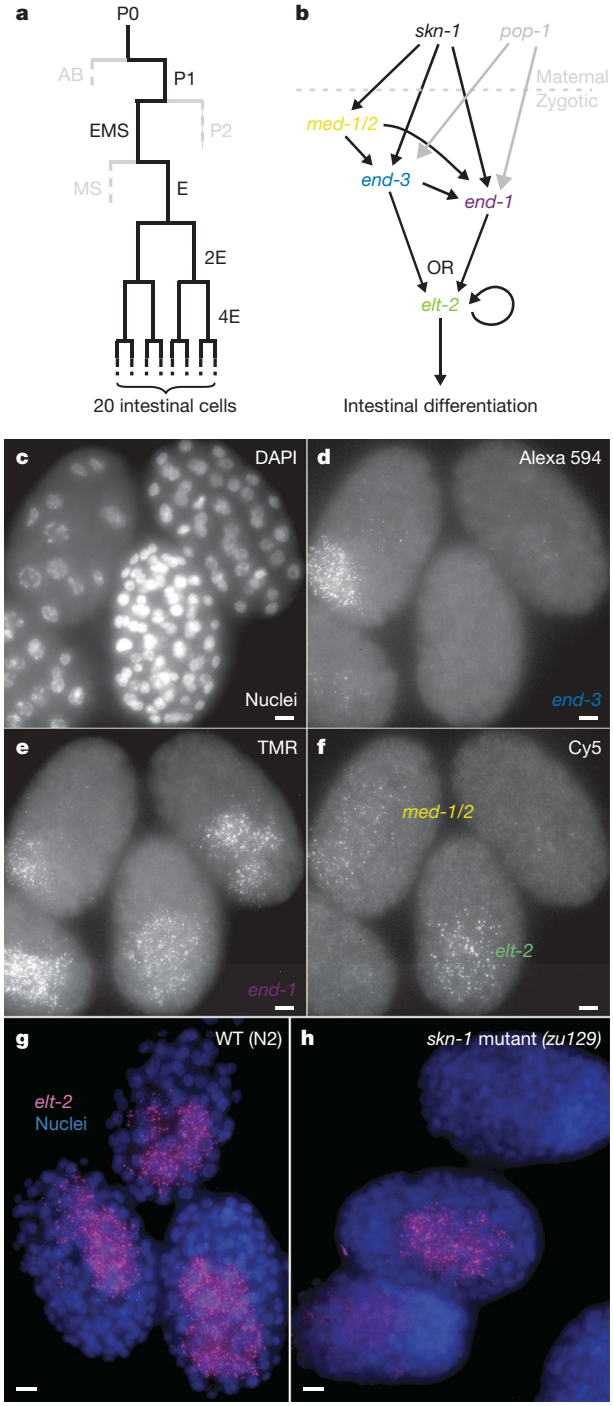

Figure 1 | Gene expression in the *C. elegans* intestinal cell fate specification network. **a**, The early embryonic lineage leading to the formation of the E cell. **b**, The gene regulatory network governing intestinal cell specification. *skn-1* and *pop-1* transcripts are maternally deposited. **c**–**f**, Visualization of single transcripts in individual wild-type (N2) embryos with DAPI as a nuclear counterstain. For Cy5, we assigned transcripts to *med-1/med-2* in embryos with less than 30 nuclei and to *elt-2* in those with more than 30 nuclei (**f**). TMR, tetramethylrhodamine. **g**, **h**, Expression of *elt-2* in wild-type (N2) (**g**) and mutant embryos harbouring the *skn-1(zu129)* allele (**h**). All scale bars are 5 μm.

In contrast, the expression of these genes was far more variable in embryos homozygous for the *skn-1* alleles *zu67*, *zu129*, or *zu135* (Supplementary Fig. 3). The expression of *elt-2* was bimodal; both the *zu67* and *zu135* alleles were more penetrant than *zu129* for loss of *elt-2* expression, in agreement with published morphological results[24]. This difference in penetrance was robust to the choice of threshold dividing OFF from ON expression (Supplementary Fig. 4).

The *skn-1* mutations also affected expression patterns of the genes upstream of *elt-2* in several ways. *med-1* and *med-2* transcripts were essentially absent, and *end-3* transcript numbers were greatly diminished (Fig. 2a and Supplementary Fig. 3), effectively removing the *med-1*/*med-2* and *end-3* nodes of the gene network. This compromised *end-1* activation, and left it as the sole activator of *elt-2* (Fig. 2b). *end-1* expression changed in two respects. First, *end-1* expression began approximately one cell cycle later than in the wild type (Supplementary Fig. 1). Second, inter-embryo variation in *end-1* expression was much higher than in wild-type nematode worms (coefficient of variation 0.68, 0.60 and 0.68 versus 0.16 for *zu129*, *zu135*, *zu67* and N2, respectively; Supplementary Figs 5 and 6).

The number of cells expressing *end-1* and *elt-2* within individual mutant embryos also varied greatly from embryo to embryo (Fig. 2c and Supplementary Fig. 3). Furthermore, the total level of *elt-2* expression correlated well with the total number of expressing cells. This suggests that each cell expressing *elt-2* produced a constant amount of transcript (around 20–30% lower than the wild type; see Supplementary Fig. 7) and that expression variability came largely from differences in the number of actively transcribing cells (Fig. 2d, bottom, and Supplementary Fig. 7), consistent with the bimodal expression pattern one would expect from a gene exhibiting self-activation. The expression of *end-1*, however, was quite variable even for a given total number of *end-1* expressing cells (Fig. 2d, top, and Supplementary Fig. 7) and even between cells in the same embryos (Supplementary Fig. 8).

end-1 must reach a threshold to activate *elt-2*

Because *elt-2* activation in these mutant embryos depended primarily if not solely on *end-1*, we hypothesized that *end-1* expression needed to reach a threshold level during a critical developmental time window to activate *elt-2*. Sub-threshold levels of *end-1* would fail to induce *elt-2* expression (Fig. 3a). To test this hypothesis, we looked for a relationship between levels of *end-1* and activation of *elt-2* in *skn-1* mutant embryos (Fig. 3b). For all *skn-1* mutants, *elt-2* expression was only found in embryos with high levels of *end-1* expression between the 65-cell and 120-cell stage, whereas both genes were highly expressed in wild-type embryos during this time. The same pattern was apparent in individual cells (Supplementary Fig. 9). After the 120-cell stage, most mutant embryos had negligible levels of *elt-2* expression (Fig. 3b, right panels). In embryos that did express high levels of *elt-2*, distributions of *end-1* and *elt-2* were similar to wild type. This is consistent with our hypothesis that *end-1* expression needs to reach a threshold, perhaps during a critical developmental window, to activate *elt-2*. The threshold itself may be caused by the self-activation of *elt-2*, with a certain amount of expression (modulated by END-1) being required to trigger the feedback loop[8].

We also examined how the number of cells expressing *end-1* influenced the decision to express *elt-2*. We found that only embryos expressing *end-1* in four or more cells expressed enough *end-1* to activate *elt-2* (Fig. 3c, d and Supplementary Fig. 10). An analysis of the dynamics of intercellular variability in cells expressing *end-1* indicated that the thresholding decision was made when there are two E cells (Supplementary Fig. 11).

The *zu67* and *zu135* alleles of *skn-1* were more penetrant for lack of *elt-2* expression than the *zu129* allele. Between the 65-cell and 120-cell stages, the distributions of *end-1* transcript number in the *zu67*, *zu129* and *zu135* strains were not significantly different ($P = 0.11$, Anderson–Darling test). However, the thresholds differed between strains, with *elt-2* activation requiring 259 and 249 *end-1* transcripts in *zu67* and *zu135* embryos compared to 143 in *zu129* embryos (Fig. 3b). This indicates that the lower penetrance in *zu129* was primarily the result of a lowered threshold (Supplementary Fig. 12).

The greatly increased variability in *end-1* expression in *skn-1* mutants could originate from several sources, including transmitted

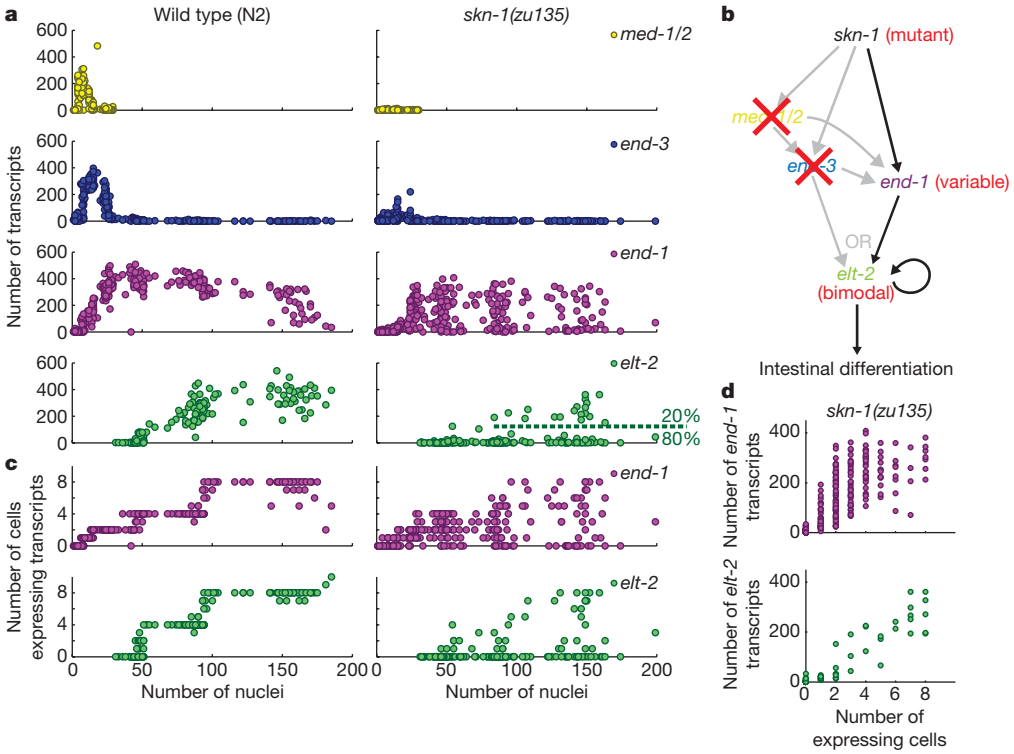

Figure 2 | Expression dynamics in wild-type and *skn-1* mutant embryos. **a**, Transcript number versus number of nuclei for a collection of randomly staged wild-type (N2) (left) and *zu135* mutant (right) early embryos. **b**, Depiction of the operation of the gut differentiation network in *skn-1* mutant embryos. **c**, Number of cells expressing *end-1* (top) or *elt-2* (bottom) within individual wild-type and *zu135* mutant embryos versus number of nuclei. **d**, Transcript number versus number of cells expressing *end-1* (top) or *elt-2* (bottom) in *zu135* mutant embryos.

variability in *skn-1* or *end-3* expression or fluctuations in the effectiveness of a POP-1-mediated Wnt signal important for activating *end-1* (refs 29–31; Fig. 1b). Analysis of *skn-1* and *end-3* mRNA levels in mutant strains argues against the first two possibilities (Supplementary Figs 6, 13–15). *pop-1* mRNA levels in the *skn-1* mutant embryos were virtually identical to those in the wild type (Supplementary Fig. 16), and POP-1 protein localization in the *skn-1* mutants was similar to that observed in the wild type (data not shown), showing that fluctuations in *pop-1* expression are unlikely to have a significant role in *end-1* expression variability.

Chromatin remodelling affects variability

Several recent studies have implicated fluctuations in chromatin state between transcriptionally active and inactive conformations as a major source of variability in gene expression[32–34]. In the intestinal specification network, *end-1* is maintained in a transcriptionally inactive state through the activity of the histone deacetyltransferase HDA-1 (ref. 30). *skn-1* activates *end-1* by recruiting the p300/CBP homologue CBP-1, which remodels the chromatin into a transcriptionally active state[35,36]. In the *skn-1* mutants, inefficient recruitment of CBP-1 to the *end-1* promoter could result in more sporadic transcriptional activation, leading to increased expression variability[32,37–39]. Thus, if the transcriptional repression by *hda-1* were relieved, this variability should decrease because the activation of *end-1* would no longer depend on the intermittent activity of CBP-1. To test this hypothesis, we measured intestinal network expression variability in *skn-1* mutant embryos in which we downregulated *hda-1* expression by RNA interference (RNAi). We found that *elt-2* expression levels increased greatly, with virtually every embryo past the 100-cell stage showing some degree of *elt-2* expression (Fig. 4a, b and Supplementary Fig. 17). This increase was due to a reduction in *end-1* expression variability and a shift in the expression distribution towards wild-type levels (Fig. 4c).

HDA-1 is a global regulator and could a priori increase *elt-2* expression in a manner independent of the increase in *end-1* expression. We think that this is not the case for two reasons. First, *end-1* and *elt-2* expression are still correlated in these embryos (Supplementary Fig. 18), strongly indicating that the increase in *elt-2* expression is directly related to the increase in *end-1* expression. Second, the expression of *med-1/med-2* and *end-3* remained low, providing evidence that the removal of *hda-1* specifically affected *end-1*. These data suggest that the proper regulation of chromatin may have a major role in controlling variability in gene expression.

Redundancy controls expression variability

Redundancy is a prominent feature of the endoderm specification network[22], and may have a role in controlling developmental errors[40]. All three *skn-1* mutations affect the expression of *end-1* both directly by hindering the transcriptional activation of *end-1* by the SKN-1 protein and indirectly by downregulating *med-1/med-2* and *end-3* (refs 19, 41, 42). To measure how much *end-1* expression variability was caused by the lack of *end-3* expression, we measured transcript numbers in a strain with an *end-3* deletion (*ok1448*)[22] (Fig. 4b, c). *end-1* expression was not delayed as it was in the *skn-1* mutants (Supplementary Fig. 1), indicating that *end-3* is not important for the initiation of *end-1* expression. We did, however, find that *end-3* mutants occasionally displayed low levels of *end-1* expression between the 40-cell and 120-cell stages, showing that some of the *end-1* expression variability in the *skn-1* mutants stems from a lack of *end-3* expression.

END-3 also acts together with END-1 in a largely redundant fashion to activate *elt-2* (ref. 22). To investigate this regulation, we measured *elt-2* expression in *end-1(ok558)* and *end-3(ok1448)* deletion mutants. Deletion of *end-1* had no discernible effects on *elt-2* expression (Supplementary Fig. 19). *end-3* mutants, however, showed variable delays in the activation of *elt-2*, although eventually, almost all embryos expressed appreciable (yet variable) levels of *elt-2* (consistent with the roughly 5% of *end-3* knockout animals that lack intestinal cells[22]). This shows that some of the variability in *elt-2*

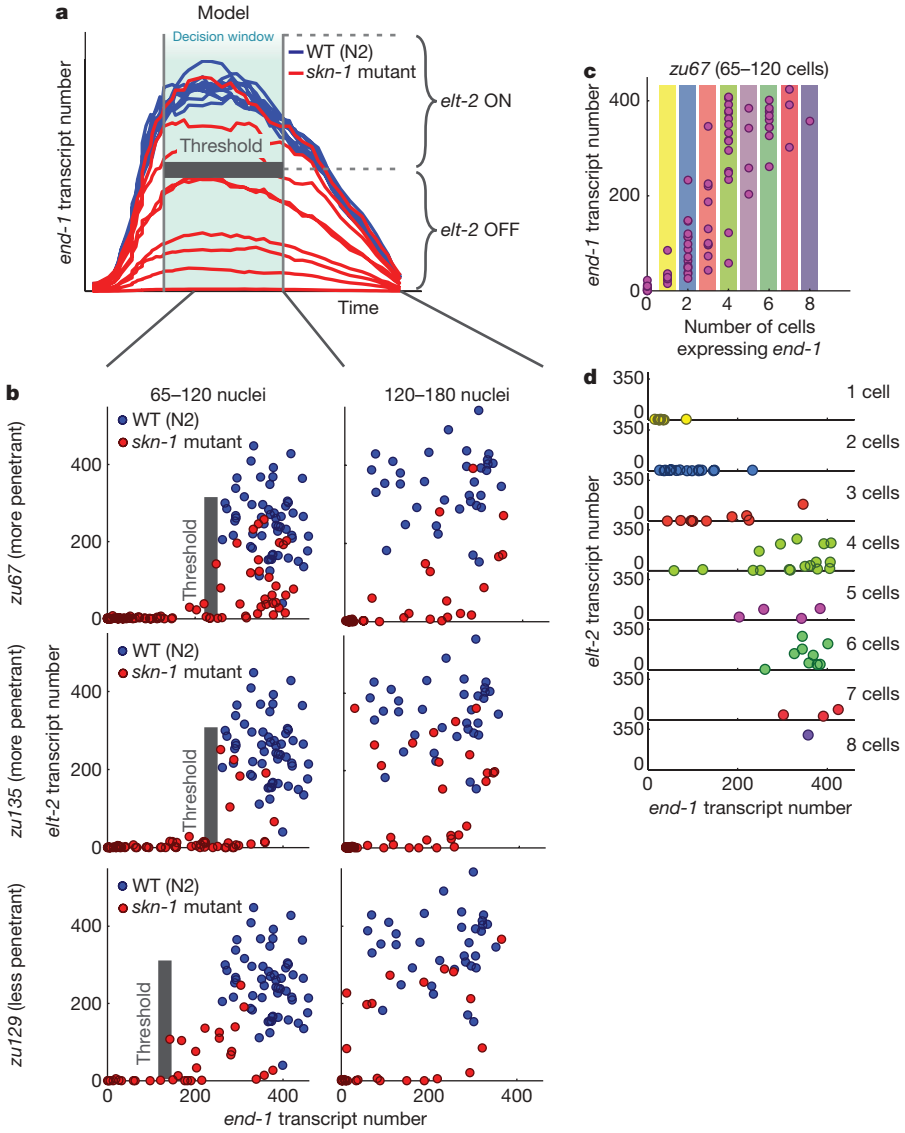

Figure 3 | High levels of *end-1* are required for *elt-2* expression in *skn-1* mutant embryos. **a**, Model in which *end-1* expression must surpass a threshold during a window of developmental time to activate *elt-2* expression. **b**, Scatter plots of *end-1* and *elt-2* transcript numbers in wild-type (N2; blue) and *skn-1* mutant embryos (red). **c**, Transcript number versus number of cells expressing *end-1* in *zu67* mutant embryos containing between 65 and 120 nuclei. **d**, Number of *elt-2* versus *end-1* transcripts in *zu67* mutant embryos (**c**) with 1–8 (top to bottom) cells expressing *end-1*.

expression in the *skn-1* mutants was due to the lack of appreciable *end-3* expression in those embryos and helps explain why some *skn-1* mutant embryos with high levels of *end-1* failed to express *elt-2*. As in the *skn-1* mutants, *end-3* mutant embryos with low levels of *end-1* expression showed low levels of *elt-2* expression (Supplementary Fig. 18). However, many more embryos had delays in *elt-2* expression than had low levels of *end-1*. Although *end-1* alone is not very efficient at activating *elt-2* expression at precisely the right time, it is able to activate *elt-2* eventually if present in sufficient quantity.

Theoretical[43,44] and experimental[45,46] work suggests that connections between different genes in a regulatory network may buffer genetic and environmental variation. In particular, removing more highly connected genes results in a greater susceptibility to variation than less connected genes. Although the intestinal differentiation network is small with many unknown interactions, our data are at least consistent with the notion that connectivity may also help buffer stochastic variability. In the intestinal specification network, *end-1* regulates the smallest number of genes whereas *end-3* and *skn-1* are progressively more connected, indicating that the amount of gene expression variability induced corresponds to the degree of connectivity of the removed node.

Concluding remarks

We have demonstrated that the incomplete penetrance of the *skn-1* mutant phenotype is a consequence of large variations in gene expression that are thresholded during development to determine cell fate[1]. Our single molecule methodology allowed us to measure these effects quantitatively, showing that mutations to genes in the network alter the topology and compromise the logic of the intestinal specification network, leading to changes in gene expression levels, variability and timing. In particular, the high variability in the mutants shows that metazoan gene expression can be highly variable and that wild-type metazoan developmental networks control those fluctuations[47]. Although inhibition of global regulators like Hsp90 can disrupt this buffering to expose hidden genetic, environmental and stochastic variation[48,49], we show that variability also arises from mutations to genes with far more specific functions, such as *skn-1* and *end-3*. Thus, random variability may have a role in driving the evolution of buffering mechanisms[50]. We propose that stochastic fluctuations in gene expression may underlie the phenotypic variation that often arises in mutant organisms even in fixed genetic and environmental contexts, and anticipate that studies like ours may help to elucidate the features of developmental networks that control the effects of underlying variation.

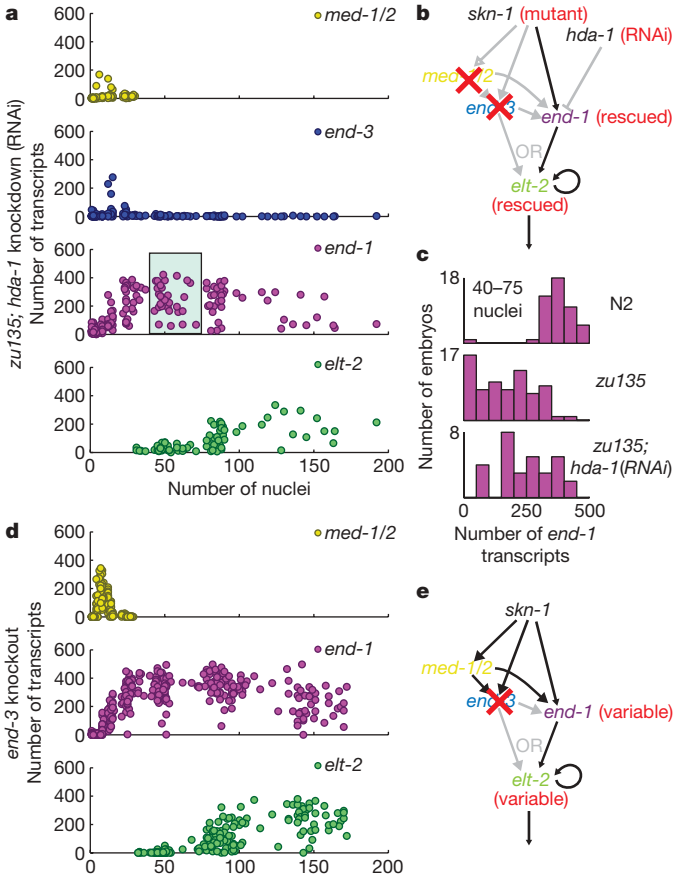

Figure 4 | Chromatin regulators and indirect network connections regulate variability in *end-1* expression. **a**, Expression dynamics in the *zu135* strain subjected to RNAi against *hda-1*. The boxed region contains embryos with 40–75 nuclei, which are further analysed in **c**. **b**, Depiction of the role of *hda-1* in the gut differentiation network. **c**, Histograms of the number of *end-1* transcripts in wild-type (top; coefficient of variation of 0.20 ± 0.057; error obtained by bootstrapping), *skn-1(zu135)* (middle; coefficient of variation of 0.69 ± 0.066) and *skn-1(zu135);hda-1(RNAi)* (bottom; coefficient of variation of 0.44 ± 0.056) embryos containing between 45 and 75 nuclei. **d**, Expression dynamics with an *end-3* deletion. **e**, Depiction of the gut differentiation network with *end-3* deleted.

METHODS SUMMARY

We collected and fixed embryos from synchronized cultures of wild-type (N2) and mutant nematodes grown at 25 °C. We maintained the *skn-1* mutant alleles as heterozygotes using a balancer containing a fluorescent protein reporter; we isolated homozygotic embryos by sorting based on fluorescence. We performed FISH on the embryos and counted the mRNAs as described previously[14]. We manually processed the images to determine the location and number of nuclei in each embryo using custom software written in MATLAB.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Strain construction. We replaced the *nT1[Unc(n754)]* translocation in the *skn-1* mutants *skn-1(zu67)*, *skn-1(zu129)* and *skn-1(zu135)* in strains EU1, EU40 and EU31, respectively[11] with an *nT1* translocation carrying the dominant marker *qIs51[myo-2::gfp]* and maintained the stocks as GFP positive *skn-1/nT1[qIs51]* heterozygotes. We also used the strains VC271 and RB1331 with genotypes *end-1(ok558)* and *end-3(ok1448)*, respectively. Both are deletion alleles from the *C. elegans* Knockout Consortium.

Worm growth, sorting and fixation. To grow large quantities of synchronized worms, we collected embryos by bleaching gravid adults, washing the embryos twice with water and then resuspending the worms in S-medium. These embryos hatched and then underwent growth arrest at the L1–L2 transition. We then plated these synchronized worms and grew them at 25 °C. For the wild-type (N2), *end-1(ok558)* and *end-3(ok1448)* strains, we grew the worms until almost all the hermaphrodites were gravid. At this point, many embryos had already been laid; to remove those later staged embryos, we ran the culture through a 40 μm cell strainer (Becton Dickinson), which retains the gravid adults while allowing the free embryos to pass through. At this point, we fixed the embryos as described in ref. 19 using solutions kept at 25 °C to minimize temperature variations, after which we performed FISH on the embryos as described below.

For the *skn-1* mutant strains (*zu67*, *zu135*, *zu129* balanced by the translocation *nT1[qIs51]* which contains a pharyngeal GFP marker), we needed to isolate large numbers of embryos that were homozygous for the mutant allele of *skn-1*. Because *skn-1* is a maternally deposited mRNA, these adults (which are GFP-negative) are viable and the effects of the *skn-1* mutations show up in their offspring. To collect *skn-1* homozygotes, we first grew a synchronized culture for 2 days at 25 °C, after which we ran the worms through a Union Biometrica BioSorter to isolate only the GFP-negative worms. This sorting procedure resulted in fewer than 1 in 500 of the resulting population being GFP positive. After sorting, we placed the homozygous mutant worms back at 25 °C for 24 h, at which point all the worms were gravid. We then fixed them as outlined above. To perform the *hda-1* RNAi on *zu135* mutant embryos, we sorted for *zu135* homozygotes using the BioSorter and then moved the worms onto plates seeded with bacteria expressing double-stranded RNA corresponding to *hda-1* once they reached the L4 stage, after which we fixed the resultant embryos as described. We also grew some *zu135* homozygotes on plates seeded with control bacteria (HT115(DE3); L4440 empty vector), finding expression patterns similar to those found on plates seeded with OP50.

FISH and imaging. We performed FISH as outlined in ref. 14. All hybridizations were performed in solution using probes coupled to either tetramethylrhodamine (TMR) (Invitrogen), Alexa 594 (Invitrogen) or Cy5 (GE Amersham). We used TMR for the probes against *end-1* mRNA, Alexa 594 for *end-3* mRNA and Cy5 for *elt-2* and *med-1/2*. The only exceptions to this labelling scheme are for the *skn-1* and *pop-1* experiments in the Supplementary Information; the fluorophores used in those experiments are described in the Supplementary figure legends. Optimal probe concentrations during hybridization were determined empirically. Imaging involved taking stacks of images spaced 0.3 μm apart using filters appropriate for DAPI, TMR, Alexa 594 and Cy5. We imaged the embryos using a Nikon TE2000 equipped with a Princeton Instruments camera and custom filter sets designed to distinguish between the different fluorophores used. During imaging, we minimized photobleaching through the use of an oxygen-scavenging solution using glucose oxidase[14]. We also reduced the out of focus light by squeezing the embryos between two coverslips, thus reducing their extent in the *z* direction.

Image analysis. We segmented the embryos manually and manually counted the nuclei in each embryo with the aid of custom software written in MATLAB (Mathworks). We discounted all embryos with greater than 200 nuclei from our analysis because such embryos are developed to the point where the key developmental decisions have already been made.

We counted the number of fluorescent spots, each of which corresponds to an individual mRNA, using the semi-automated method described in ref. 14. We estimate our mRNA counts to be accurate to within 10–20%. We also counted the number of cells expressing either *end-1* or *elt-2* by manually selecting positive cells in every embryo.