A bistable autoregulatory module in the developing embryo commits cells to binary fates

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Abstract  Positive autoregulation has been repeatedly proposed as a mechanism for cells to adopt binary fates during embryonic development through bistability. However, without quantitatively determining their parameters, it is unclear whether the plethora of positive autoregulatory modules found within developmental gene regulatory networks are actually bistable. Here, we combine in vivo live imaging with mathematical modeling to dissect the binary cell fate dynamics of the fruit fly pair-rule gene *fushi tarazu* (*ftz*), which is regulated by two known enhancers: the early (non-autoregulating) element and the autoregulatory element. Live imaging of transcription and protein concentration in the blastoderm revealed that binary Ftz cell states are achieved as Ftz expression rapidly transitions from being dictated by the early element to the autoregulatory element. Moreover, we discovered that Ftz concentration alone is insufficient to activate the autoregulatory element, and that this element only becomes responsive to Ftz at a prescribed developmental time. Based on these observations, we developed a dynamical systems model, and quantitated its kinetic parameters directly from experimental measurements. Our model demonstrated that the *ftz* autoregulatory module is indeed bistable and that the early element transiently establishes the content of the binary cell fate decision to which the autoregulatory module then commits. Further analysis in silico revealed that the autoregulatory element locks the Ftz expression fate quickly, within 35 min of exposure to the transient signal of the early element. Overall, our work confirms the widely held hypothesis that autoregulation can establish developmental fates through bistability and, most importantly, provides a framework for the quantitative dissection of cellular decision-making based on systems dynamics models and real-time measurements of transcriptional and protein dynamics.
1 Introduction

One of the central questions in developmental biology concerns how cells precisely and irreversibly adopt distinct cellular fates. It has been argued that cells assume their unique gene expression profiles through a sequence of decisions among branching paths (Zernicka-Goetz et al., 2009; Soldatov et al., 2019), most famously encapsulated by C. H. Waddington’s “epigenetic landscape” of peaks and valleys delineating the possible trajectories that a cell can follow (Waddington, 1957). Genetic networks that lock a cell into one of these trajectories may be thought of as “memory modules” that guide cells through valleys in the landscape to their ultimate fates. In the simplest case, where a decision is made between two alternative developmental fates, the memory module is binary and is often referred to as a switch. The state of the switch is set by the action of transient upstream regulatory signals.

Several genetic motifs, such as autoactivation and mutual repression, have been identified that are capable of maintaining binary cell fates (Alon, 2007; Peter and Davidson, 2015). However, the mere presence of a motif is insufficient to guarantee that a network can remember its expression state once upstream regulators have degraded. The ability to lock onto high or low expression levels results from bistability (Box 1), a systems-level property that depends upon the quantitative details of the kinetics of the involved chemical reactions (Ferrell, 2002; Angeli et al., 2004; Graham et al., 2010).

Despite the widespread invocation of bistability to explain the stable and irreversible determination of cellular fates (Peter and Davidson, 2015), relatively little quantitative data exist to confirm bistability in gene expression modules within developing embryos. Previous studies in cell culture and fixed embryos have provided evidence for the existence of bistability in hematopoietic differentiation (Laslo et al., 2006; Kueh et al., 2013), the Shh network (Lai et al., 2004), the vertebrate hindbrain (Bouchouca et al., 2013), between the BMP and FGF morphogens (Srinivasan et al., 2014), and within the Notch-Delta signaling system (Sprinzak et al., 2010). Quantitative evidence for multistability in fruit fly embryos has also been derived from fitting the parameters of high-dimensional network models to measurements in fixed tissue (von Dassow et al., 2000; Jaeger et al., 2004; Lopes et al., 2008; Manu et al., 2009; Papatsenko and Levine, 2011; Verd et al., 2017).

While these models are capable of reproducing the observed phenomenology, there is often no guarantee that the optimal set of inferred parameter values reflects actual biophysical quantities (Gutenkunst et al., 2007; Cotterell and Sharpe, 2010; Villaverde et al., 2015; Wieland et al., 2021). Thus, it is important to verify that the conclusions drawn from computational modeling and in vitro experiments apply to developmental systems in vivo in the context of models that quantitatively capture the molecular interactions that underlie the process of cellular decision making. To the best of our knowledge, evidence for the bistability of a genetic module based on these molecular interactions in an intact multicellular organism has not yet been demonstrated.

The early development of the fruit fly Drosophila melanogaster is an ideal model system for studying binary cell fate decision making. Specifically, the mapping of the regulation of fushi tarazu (ftz), one of the Drosophila pair-rule genes that forms seven discrete stripes at the cellular blastoderm stage prior to gastrulation (2.5 - 3.5 hours after fertilization; Nüsslein-Volhard and Wieschaus, 1980; Hafen et al., 1984; Wakimoto et al., 1984; Weiner et al., 1984; Hiromi et al., 1985), has suggested that this gene is capable of autoactivation (Hiromi and Gehring, 1987). These studies showed that two main enhancers—the early, or zebra, element and the autoregulatory element—dictate Ftz protein expression during early embryogenesis (Figure 1A; Hiromi and Gehring (1987)). The early element responds to upstream transcription factors such as the gap genes to establish the initial expression pattern of seven stripes (Dearolf et al., 1989). This element is functionally distinct from the autoregulatory element, which contains multiple Ftz binding sites that allow Ftz to activate its own expression (Pick et al., 1990; Schier and Gehring, 1992, 1993). This autoactivation...
network motif is theoretically capable of exhibiting bistability and has therefore been hypothesized to act as a binary memory module (Alon, 2007; Xiong and Ferrell, 2007).

Whether a cell possesses a memory module determines whether observed states of gene expression are transient in the absence of continued external signaling, or whether these states can be locked into permanent cell fates that can be maintained without further intervention. Specifically, if the autoregulatory module is bistable, then high ftz expression driven by the transient presence of upstream factors is stabilized by the autoregulatory element into a permanent cell fate (Figure 1B, case 1), even once those factors degrade (or until further regulatory mechanisms intervene). If, instead, the autoregulatory element is monostable, then the observed separation of Ftz concentration into high and low levels persists only as long as upstream factors are present to regulate expression. In their absence, Ftz expression would revert to a single fate for all cells (Figure 1B, case 2). It is important to note, however, that in this case, the transiently high or low trajectory of Ftz concentration could still be instructive for regulating downstream genes.

Here, we characterize the ftz autoregulatory module in vivo through quantitative real-time measurements in living fruit fly embryos. We focus on the anterior boundary of stripe 4, the only Ftz stripe that has been shown to be driven exclusively by the early and autoregulatory elements and not by other enhancers in the gene's vicinity (Schroeder et al., 2011; Graham et al., 2021). We observe that Ftz expression separates into high and low levels at the blastoderm stage during the
20 min prior to gastrulation, concurrent with a transition in regulatory control from the early to
the autoregulatory element. We discover that autoregulation is triggered at a specific time point in
development—presumably through the action of “timer genes” (Clark and Akam, 2016; Clark and
Peel, 2018; Clark et al., 2022)—rather than through a readout of Ftz concentration alone. Based on
these observations, we develop a dynamical systems model and quantitate its parameters from
simultaneous real-time measurements of ftz transcription and Ftz protein dynamics in single cells
of living embryos. Our model predicts binary Ftz expression levels at gastrulation with high accu-

curacy and demonstrates that, indeed, the ftz autoregulatory module is bistable. We conclude that
the ftz autoregulatory element acts as a memory module to commit cells to binary fates that are
otherwise transiently defined by the early element, thereby validating a long-standing hypothesis
in developmental and systems biology. Simulations further make it possible to quantitatively de-
fine a developmental commitment window, which shows that the autoregulatory module requires
about half an hour to establish a memory of the transient signal from the early module. Thus, our
work provides a framework for the dissection of other regulatory modules in the gene regulatory
networks that dictate development based on this interplay between dynamical systems models
and real-time experiments.

2 Results

2.1 Binary cell states of Ftz expression are established in early development

To understand the role of positive autoregulation in deciding Ftz expression levels, we first sought
to visualize and track Ftz protein dynamics over time. We used CRISPR-mediated recombination
(Gratz et al., 2015) to fuse a LlamaTag, a fluorescent probe that reports on the fast protein dynamics
that characterize early embryonic development, to the C-terminus of the endogenous Ftz protein
(Figure 2A; Bothma et al. (2018)). An examination of the fluorescently labeled Ftz protein in the
early embryo shows that, around 15 min before gastrulation, Ftz protein is expressed in a seven-

stripe pattern with clear, smooth boundaries (Figure 2B, left). This expression pattern refines over
the following 15 min into sharp stripe boundaries by the start of gastrulation (Figure 2B, right). The
result shows that cells express either high or low levels of Ftz protein, as pictured in Figure 2C for
the anterior boundary of stripe 4, consistent with results from previous studies (Schier and Gehring,
2011; Clark, 2017; Bothma et al., 2018).

Our live imaging measurements allowed us to quantitatively examine the dynamics with which
binary cell states are established by calculating the Ftz protein distribution in individual nuclei at
different time points in development (Figure 2D). Our analysis revealed that the expression level
is initially unimodal across all cells (Figure 2D, top and E) and then evolves into a bimodal distri-

bution within 15 min (Figure 2D, bottom and E). Consistent with our qualitative observations in
Figure 2B and C, cells at these later times can be quantitatively classified into distinct “high-Ftz”
and “low-Ftz” cell states using a single threshold (Figure 2D, bottom and E), which indicates that
binary cell states are already established prior to the onset of gastrulation. Moreover, though the
two cell states are clearly distinguishable from each other, we observed that there is significant
cell-to-cell variability within both states. Specifically, the single-nucleus Ftz protein distribution for
high-Ftz levels spans more than a two-fold range (Figure 2D, bottom and E).

Previous studies have established that autoregulation plays a key role during ftz expression: a
lack of the ftz autoregulatory element or mutated Ftz binding sites within the element result in the
loss of Ftz expression at later developmental stages (Hiromi and Gehring, 1987; Schier and Gehring,
1992). However, it is unclear at what developmental time ftz autoregulation is initiated in response
to the Ftz expression stemming from the early element. Specifically, is the autoregulatory element
active before stripes of Ftz expression emerge, or is autoregulation invoked after the early element
has already established this pattern? To distinguish between these two scenarios, we decoupled
Box 1. Bistability

A simplified dynamical systems model of the ftz autoregulatory element that ignores the dynamics of mRNA production describes the rate of change in Ftz concentration over time as

\[
\frac{dP(t)}{dt} = f(P(t)) - \gamma P(t) \tag{1}
\]

where \( P \) is Ftz concentration, \( f(P) \) is the gene regulatory (or input-output) function that describes how input Ftz concentration controls the rate of Ftz production, and \( \gamma \) is the Ftz degradation rate. Since Ftz promotes its own production, \( f(P) \) increases with \( P \).

In the long term, Ftz concentrations will tend toward stable steady states, or attractors, for which (by definition) the change in concentration over time goes to 0 (i.e., \( \frac{dP}{dt} = 0 \)). Attractors are stable, meaning that if Ftz concentration is perturbed slightly away from the attractor, it will eventually return to the attractor. However, it is also possible to have unstable steady states for which, after a small perturbation, Ftz concentration will evolve away from the steady state. For the system in Eq. 1, a steady state \( P^* \) will solve

\[
\gamma P^* = f(P^*) \tag{2}
\]

meaning that all steady states can be found graphically as the intersections between a line of slope \( \gamma \) and the function \( f \). In our case, the gene regulatory function \( f \) is a sigmoidal function (red curves in Figure B1), such that there are between 1 and 3 intersections of the total degradation rate \( \gamma P \) with the Ftz production rate \( f(P) \). Then the autoregulatory module is either monostable, meaning it possesses one attractor, or bistable, meaning it possesses two attractors and one unstable steady state between these attractors. Since the number of intersections depends on the shape of \( f \) and the slope \( \gamma \), the exact parameter values are crucial for determining the possible behaviors that can be exhibited by the autoregulatory module.

![Figure B1](https://example.com/figB1)

**Figure B1.** Above, steady states are identified through the intersection of the production rate \( f(P) \) with the total degradation rate \( \gamma P \) where \( P \) is the Ftz concentration. Below, vector fields show the direction Ftz concentration will evolve over time. An equivalent graphical test also exists for 2D dynamical systems models where mRNA is modeled explicitly; see Section S1.3.

We will also consider controlled systems, i.e., dynamical systems that have an additional regulatory input. In this case, this input corresponds to the Ftz concentration produced by the early element, \( P_{early} \), such that the dynamics of Ftz production by the autoregulatory element are given by

\[
\frac{dP_{late}(t)}{dt} = f(P_{late}(t) + P_{early}(t)) - \gamma P_{late}(t) \tag{3}
\]

Since the early element shuts down over time (that is, \( P_{early}(\infty) = 0 \)), the steady states \( P_{late} \) in Eq. 3 are the same as those for \( P \) in Eq. 1. However, the transient dynamics of \( P_{early} \) shape the trajectory \( P_{late} \) will take and determine which steady state Ftz will ultimately reach.
Figure 2. Binary cell states are rapidly established prior to gastrulation. (A) A fusion of endogenous Ftz to a LlamaTag makes it possible to visualize the highly dynamic Ftz protein pattern in the early fly embryo. Once Ftz protein is translated in the cytoplasm, LlamaTag binds to maternally deposited EGFP and is transported into the nucleus, increasing nuclear fluorescence to produce a direct readout of Ftz protein concentration. (B) Snapshots from a movie capturing Ftz protein concentration dynamics. The anterior side of the embryo is oriented towards the left, and the time is given relative to gastrulation. (C) Ftz expression along the anterior boundary of stripe 4 shows a discrete transition between distinct cell states. (D) Histograms of single-nucleus fluorescence values at different developmental time points show that a single threshold can be used to classify cells into “high-Ftz” and “low-Ftz” cell states prior to gastrulation. (E) Single-cell trajectories of nuclei at the anterior boundaries of Ftz stripe 4. Green and red lines are averages for nuclei determined to ultimately have “high-Ftz” and “low-Ftz” levels at gastrulation, respectively, as defined in (D).

We observed that the early element already drives a relatively constant gene expression level around 20 min prior to gastrulation (Figure 3B and C). Then, at 15 min before gastrulation, its transcriptional activity decreases significantly, resulting in a 60% reduction within the next 20 min of development (Figure 3B and C). Conversely, autoregulation is initiated 20 min prior to gastrulation, with its activity increasing until gastrulation starts (Figure 3C). This transition between the early and autoregulatory elements occurs while binary cell states are being established (Figure 2). Since autoregulation becomes the dominant driver of Ftz expression after this transition, it is likely that autoregulation plays a key role in Ftz-mediated decision-making prior to gastrulation.
Figure 3. A sequential transition in Ftz regulation from the early to the autoregulatory element occurs during the establishment of discrete cell states. (A) Imaging transcriptional dynamics of the early and autoregulatory elements using the MS2 system. Maternally deposited MS2 coat protein (MCP) fused to mCherry binds to MS2 stem-loops in the nascent RNA of the reporter construct. (B) Snapshots of sites of nascent transcript formation labeled by MS2 from reporters of the early and autoregulatory elements at different time points reveal that transcription from the early element is reduced significantly as gastrulation approaches and that transcription driven by the autoregulatory element increases shortly before gastrulation. (C) Quantification of the transcriptional activity reported by the MS2 fluorescence from the early (\(N = 3\) embryos) and autoregulatory (\(N = 7\) embryos) elements as a function of time confirms that, within 20 min, ftz gene expression transitions from originating mainly from the early element to being dominated by the autoregulatory element. MS2 traces are smoothened using a moving average of 5 min. Error bars shown indicate standard errors over multiple embryos.

### 2.2 ftz autoactivation is triggered at a specific developmental time

The tight transition between the early and autoregulatory elements that occurs within 20 min (Figure 3) could be indicative of autoactivation being initiated by the increase in Ftz concentration driven by the early element. However, autoactivation could also be triggered by upstream factors at a specific developmental time, regardless of the Ftz level at that time point.

To distinguish between these two scenarios, we measured the gene regulatory function—the input-output function describing how the input Ftz concentration dictates the output rate of ftz transcription—of the ftz autoregulatory element at distinct developmental times. If autoactivation is solely initiated by Ftz produced by the early element, the regulatory function should remain constant throughout development. On the other hand, if autoactivation is triggered through an independent mechanism—such as activation mediated by other transcription factors at a given developmental time—then the ftz autoregulatory element should be unresponsive to input Ftz protein before this developmental time point.

We measured the gene regulatory function of the autoregulatory element by constructing an experimental system that allows for the simultaneous monitoring of Ftz concentration and the corresponding autoregulatory activity. We used the tagged endogenous Ftz protein as the input and introduced a transgenic reporter with MS2 loops under the control of the ftz autoregulatory element as the output (Figure 4A). Live imaging of the anterior boundary of Ftz stripe 4 (Figure 4B) showed that, initially, around 25 min prior to gastrulation, both Ftz expression and the autoregulatory response were relatively low, and later increased as development progressed. Just before gastrulation, the Ftz protein pattern refined into a discrete boundary, with the ftz autoregulatory...
Figure 4. Two color live-imaging reveals that ftz autoregulation is initiated at a specific developmental time. (A) Two-color tagging permits in vivo simultaneous visualization of input Ftz protein concentration using a LlamaTag and output autoregulatory transcriptional dynamics using a reporter carrying the MS2 system. (B) Representative frames from live-imaging data. Green and magenta channels correspond to Ftz concentration and the transcriptional output from the ftz autoregulatory element, respectively. Dark gray and light gray outlines correspond to cells expressing high or low levels of Ftz protein, respectively. (C) Illustrative single-cell trace of Ftz protein and autoregulatory activity. Green and magenta lines correspond to the Ftz protein and transcriptional activity of the autoregulatory element, respectively. Both protein and MS2 traces are smoothened using a moving average of 1 min. (D) Experimentally measured gene regulatory function of the ftz autoregulatory element between -10 min to -5 min relative to gastrulation. Grey points correspond to simultaneous measurements of Ftz and MS2 fluorescence at individual time points from single-cell traces at the anterior boundary of stripe 4 ($N = 211$ nuclei, example traces shown in Figure S1). These points were grouped into quantiles, and a Hill function (red line) was fit to the quantile means. (E) The autoregulatory input-output function evolves over time, as the ftz autoregulatory element transitions from an unresponsive to a responsive state within 15 min, indicating that Ftz autoregulation is initiated through a developmental time-based mechanism. Error bars shown indicate standard errors. All data are from $N = 7$ embryos.
response clearly following the stripe boundary (Figure 4B).

To calculate the regulatory function, we restricted our analysis to the cells at the anterior boundary of stripe 4 as a means to minimize the influence of other position-dependent transcription factors that might also contribute to ftz autoactivation (Schier and Gehring, 1993). We first extracted two rows (high and low) of boundary cells. Then, we separated the input Ftz concentration and output transcription from the autoregulatory element in the data corresponding to each individual cell (Figure 4C; Figure S1) into ten quantiles and fit a Hill function to the quantile averages to get the gene regulatory function of ftz autoregulation within a defined temporal range (for example, -10 min to -5 min for Figure 4D). Our analysis revealed a sharp regulatory relationship between Ftz protein and autoregulatory response, with a Hill coefficient of 3.3±0.8 (Figure 4D). Such Hill coefficients are comparable to those estimated in the context of autoactivation in vertebrate hindbrain development (Bouchoucha et al., 2013) as well as those observed in simpler regulatory motifs that do not feature feedback (Gregor et al., 2007).

We repeated the process described above at multiple developmental times to analyze how the regulatory function for ftz autoactivation evolves over time. The results, shown in Figure 4E, revealed that the regulatory function is clearly distinct at different time points. Specifically, initially around -25 min to -20 min, the ftz autoregulatory element is effectively unresponsive to input Ftz protein (Figure 4E, red line). However, the element progressively transitions to a fully responsive state within 15 min (Figure 4E, green line). The observed temporal evolution of the autoregulatory element’s responsiveness to Ftz protein is a clear indication that the autoregulatory element is not always primed to respond to input Ftz protein and that, instead, its expression is triggered at a specific developmental time, presumably by upstream transcription factors.

### 2.3 Mathematical modeling quantitatively predicts Ftz concentrations

As we argued in Figure 1B, the fact that Ftz can exhibit a high state at one point in time does not necessarily imply that this high state will persist in the absence of upstream regulation. Determining whether the autoregulatory module is bistable and hence possesses developmental memory requires turning the schematic shown in Figure 1A into an explicit mathematical model with empirically determined parameter values. To that end, we first developed a dynamical systems model for the full Ftz regulatory system (including both the early and autoregulatory elements) to verify whether we could accurately recapitulate experimental results in silico.

Our measurements revealed that the autoregulatory element is unresponsive to Ftz concentration until about $t_{on} = -20$ min (Figure 4E). Once the element becomes responsive, we can describe the ftz autoregulatory module using the dynamical systems model given by

$$
\begin{align*}
\frac{dR_{late}}{dt} &= cf(P_{early}(t) + P_{late}(t)) - \gamma R_{late}(t), \\
\frac{dP_{late}}{dt} &= aR_{late}(t) - \gamma_p P_{late}(t)
\end{align*}
$$

where $R_{late}(t)$ and $P_{late}(t)$ are the mRNA and protein concentrations produced by the autoregulatory module, $\gamma_R$, $\gamma_P$ are the decay rates of mRNA and protein, respectively, and $a$ is the translation rate. $f(P)$ is the gene regulatory function measured for the transgene and shown in Figure 4D that describes the output rate of mRNA production as a function of the input Ftz concentration. Since both the early and autoregulatory elements drive Ftz expression, the gene regulatory function depends on $P_{total}(t)$ which is the sum of the protein contributions from the early ($P_{early}(t)$) and autoregulatory ($P_{late}(t)$) modules. $c$ is a scaling factor between the transcriptional output of the endogenous locus and the gene regulatory function, which was measured for a transgene (Figure 4D). The first equation in the system shown in Eq. 4 then describes the dynamics of mRNA produced from the autoregulatory element as a result of its transcriptional activity (first term on the right-hand side) and mRNA degradation (second term on the right-hand side). Further, the second equation describes
Figure 5. Mathematical modeling accurately predicts binary Ftz expression states at gastrulation. (A) Example trajectories for the mRNA production rate $r(t)$, mRNA concentration $R_{\text{early}}(t)$, and protein concentration $P_{\text{early}}(t)$ from the early element, as described by Eq. 5. (B) Representative traces for three nuclei comparing empirical total Ftz trajectories (dark green) to simulated trajectories (green) comprising contributions from both the early (blue) and autoregulatory (red) elements. Gray dashed line marks the experimentally determined threshold used to classify cells as high or low Ftz expression states at gastrulation (Figure 2D). The plotted nuclei correspond to the circled and labeled points in panel C. Note that the simulations extend well past the time that we can obtain experimental measurements. (C) Each nucleus has a set of initial conditions ($r(t_{\text{on}}) = r_0$, $R_{\text{early}}(t_{\text{on}}) = R_0$, $P_{\text{early}}(t_{\text{on}}) = P_0$) for the dynamics of the early element. The blue surface separates those nuclei (circles) that are predicted to express low levels of Ftz at gastrulation (below the surface) from those that are predicted to express high levels of Ftz (above the surface). Color intensity corresponds to the Ftz concentration at gastrulation. Downward-facing red triangles indicate false positives (predicted high at gastrulation, but experimentally determined to be low) and upward-facing red triangles indicate false negatives (predicted low, but experimentally determined to be high). Results are shown for $N = 118$ nuclei from 3 embryos at the anterior boundary of stripe 4, with model parameters given in Table S1.

After time $t_{\text{on}}$, in addition to mathematically describing the expression dynamics of the autoregulatory element, we can also model the contribution of the early element to Ftz expression as a dynamical system. In particular, our empirical measurements of the early element transcription rate using the MCP-MS2 system revealed that its transcription rate $r(t)$ follows an approximately exponential decay with a with a decay constant $\beta^{-1}$ of about 21 min (Figure 3C; Figure 54; Section S2.3). We can then represent the mRNA $R_{\text{early}}(t)$ and protein $P_{\text{early}}(t)$ produced from the early element through the translation of the mRNA produced by this element (first term on the right-hand side) and degradation (second term on the right-hand side). Finally, as indicated in Eq. 4, we assume that the autoregulatory element is only active for $t \geq t_{\text{on}}$. We note, however, that our model produced nearly identical results whether we assumed that this transition to full responsiveness occurred instantaneously at time $t_{\text{on}}$, or whether we assumed a gradual increase in responsiveness over time (Section S3.1).

The protein resulting from the autoregulatory element through the translation of the mRNA produced by this element (first term on the right-hand side) and degradation (second term on the right-hand side). Finally, as indicated in Eq. 4, we assume that the autoregulatory element is only active for $t \geq t_{\text{on}}$. We note, however, that our model produced nearly identical results whether we assumed that this transition to full responsiveness occurred instantaneously at time $t_{\text{on}}$, or whether we assumed a gradual increase in responsiveness over time (Section S3.1).
using the linear system of equations

\[
\begin{align*}
\frac{dr}{dt} &= -\beta r(t) \\
\frac{dR_{\text{early}}}{dt} &= r(t) - \gamma_R R_{\text{early}}(t) \\
\frac{dP_{\text{early}}}{dt} &= aR_{\text{early}}(t) - \gamma_P P_{\text{early}}(t).
\end{align*}
\]

(5)

where the equation for \( \frac{dr}{dt} \) has been introduced to model the approximately exponential decay of the rate of transcription of the early element after the onset time \( t_{on} \). Here, \( R_{\text{early}} \) dynamics are dictated by the transcription rate \( r(t) \) and the mRNA degradation rate. The early protein \( P_{\text{early}} \) is determined by the translation of the mRNA stemming from the early element as well as by protein degradation. Note that, regardless of the choice of parameter values or initial conditions, \( P_{\text{early}}(t) \) converges to 0 as \( t \) goes to infinity (Section S1.2), indicating that signaling from upstream factors is transient.

The behavior of the \( ftz \) regulatory system depends crucially on the quantitative values of the kinetic rates describing the molecular interactions within our model. For those parameters that were not already present in the literature, we carried out a set of experiments designed to directly measure these free parameters as described in detail in Section S2. First, the gene regulatory function \( f(P) \) was measured by averaging the traces obtained from simultaneous imaging of endogenous Ftz protein and the corresponding autoregulatory response as previously shown in Figure 4D. Second, the translation rate \( a \) was found by simultaneously measuring the rate of transcription and the resulting protein concentration in a transgenic construct where \( ftz \) mRNA was labeled with MS2 and the resulting Ftz protein tagged with a LlamaTag (Figure S3). Third, the decay in the transcription rate of the early element over time \( \beta \) was determined by fitting an exponential function to the transcriptional dynamics of the early element construct (Figure S4). Fourth, the scaling factor \( c \) was inferred by systematically comparing simulated to measured traces in the regime where the gene regulatory function was saturated and mRNA production rate was at its maximum (Figure S5).

Finally, the mRNA and protein decay rates \( \gamma_R \) and \( \gamma_P \) were drawn from existing measurements in the literature. All parameter values are reported in Table S1.

If our model of the \( ftz \) regulatory system is accurate, then for each nucleus along the anterior boundary of stripe 4, we should be able to predict Ftz expression state at gastrulation based only on measurements at time \( t_{on} = -20 \) min. Specifically, at this point, each nucleus will have a different initial expression rate, mRNA level, and protein level from the early module given by \( r(t_{on}) = r_{on} \), \( R_{\text{early}}(t_{on}) = R_{on} \) and \( P_{\text{early}}(t_{on}) = P_{on} \), respectively. The autoregulatory element, however, will not have produced any mRNA or protein prior to -20 min, so the initial conditions for the autoregulatory module will be \( R_{\text{late}}(t_{on}) = 0 \) and \( P_{\text{late}}(t_{on}) = 0 \).

Each set of initial conditions \( (r_{on}, R_{on}, P_{on}) \) for the early module defines a trajectory for \( P_{\text{early}}(t) \) (Figure 5A) that will in turn drive expression from the autoregulatory module. The result is a unique overall trajectory for total Ftz concentration \( P_{\text{total}}(t) = P_{\text{early}}(t) + P_{\text{late}}(t) \) (Figure 5B) for each nucleus.

Hence, for a given set of initial conditions, we can simulate the full dynamical system starting at \( t_{on} \) and see whether total Ftz concentration exceeds a threshold at gastrulation (i.e., \( P_{\text{early}}(t = 0) + P_{\text{late}}(t = 0) > P_{\text{thresh}} \), where \( P_{\text{thresh}} \) is empirically determined as shown in Figure 2D) in order to predict the Ftz expression state of the nucleus at gastrulation. Moreover, since increasing any one of the initial conditions can only increase total Ftz, if we plot all possible sets of initial conditions \( (r_{on}, R_{on}, P_{on}) \), then a single smooth surface separates the sets that result in high Ftz at gastrulation (above the surface) from the sets that result in low Ftz at gastrulation (below the surface), as visualized in Figure 5C.

We used the smooth surface separating high and low Ftz states in our model to directly predict
we next analyzed the behavior of the early and autoregulatory modules separately, with the goal of uncovering whether the autoregulatory module is bistable and, as a result, retains a long-term memory of these Ftz levels.

Having established that our model accurately predicts transient Ftz expression state at gastrulation, we next analyzed the behavior of the early and autoregulatory modules separately, with the goal of uncovering whether the autoregulatory module is bistable and, as a result, retains a long-term binary memory of these Ftz levels.

We first performed a test of the autoregulatory module to ascertain whether it is bistable in the absence of Ftz contribution from the early element \( P_{\text{early}}(t) = 0 \). Such bistability would enable the module to maintain a high or low expression state even once upstream regulatory factors binding the early element have degraded. Following the procedure described in Box 1 and Section S3.4, we identified the steady states where mRNA and protein concentrations no longer change in time. Our analysis revealed that, indeed, the empirically determined model parameters set the autoregulatory module in a bistable regime (Figure 6A) characterized by the presence of two stable steady states corresponding to low and high Ftz values. This result indicates that the \( ftz \) autoregulatory module is capable of maintaining high or low levels of \( ftz \) expression indefinitely.

Since our model predicts that the autoregulatory element is capable of remembering Ftz expression levels, the question thus arises of whether the module actually becomes responsive to Ftz concentration in time to lock the transient state observed in the blastoderm into a permanent cell fate. Otherwise, if the bistable module becomes responsive too late, cells could transiently express high Ftz at gastrulation without committing to stably expressing high Ftz in the long term.

To distinguish between these two scenarios, we compared the binary classification of nuclei into high or low Ftz expression states at gastrulation as introduced in the previous section with the final high or low expression fate predicted by the model in steady state (Figure 6B). Similarly to the case of binary classification, the regions of parameter space \( (r_0, R_0, P_0) \) resulting in high fates are separated from the region of parameters resulting in low fates by a surface called the switching separatrix (Sootla et al., 2016), so named because if the initial conditions of a cell are above the surface, then the bistable autoregulatory module will switch on. The initial conditions for all nuclei at the anterior of stripe 4 are plotted in Figure 6C alongside the switching separatrix (red surface) and the surface for transient binary classification of Ftz state at gastrulation (blue surface).

If we restrict our analysis to the “best predicted” nuclei (expression state correctly classified at gastrulation and with relatively low cumulative error between simulation and empirical measurement over time; see Section S3.1), 93.7% (74 of 79 nuclei) were predicted to maintain their binary
Figure 6. Quantitative mathematical model reveals that the ftz autoregulatory module is bistable. (A) The intersection between Ftz degradation rate and the effective Ftz production rate (i.e., late Ftz production rate adjusted for late ftz mRNA production and decay rates) reveals that the autoregulatory module is bistable, as described in Box 1 and Section S1.3. (B) Most cells that express high Ftz state at gastrulation reach the high fate at steady state (cell 3), but some transiently express high Ftz at gastrulation before ultimately reaching the low fate (cell 2). The threshold (dashed gray line) is only used to determine state at gastrulation; fate is decided by which of the two stable steady states is approached by the system in simulation at long times. (C) The early module dictates the transient dynamics of Ftz as well as the fate it is predicted to adopt at infinite time once the autoregulatory element has reached steady state. The blue surface is repeated from Figure 5 and separates nuclei into low (below surface) and high (above surface) Ftz state at gastrulation. The red surface (switching separatrix) separates nuclei predicted to adopt the low Ftz fate (black circles) from those predicted to adopt the high Ftz fate (green circles), where by “fate” we mean expression level at infinite time (steady state). Nuclei between the blue and red surface (blue circles) are considered transiently high in that they express high Ftz at gastrulation but are predicted to adopt the low Ftz fate. Data are plotted for \( N = 118 \) nuclei from 3 embryos. Red triangles indicate false negatives (upward) and false positives (downward) as determined from classification at gastrulation. (D) Results from a representative embryo show that the experimentally measured stripe pattern (left) is recapitulated by simulation (middle). A stripe pattern is still evident at gastrulation even from the predicted early Ftz concentration alone (right). Nuclear intensities at all time points are normalized to the predicted steady-state high Ftz concentration. Red “x”s denote nuclei with a transiently high Ftz state at gastrulation; triangles denote false positives (downward) and false negatives (upward). Parameters for simulation are as given in Table S1.
state and adopt the corresponding Ftz expression fate in steady state, as exemplified by cell 3 in Figure 6B. The remaining 6.3% of nuclei (5 of 79), despite having a high Ftz expression state at gastrulation, were predicted to drop to a low Ftz fate in steady state as shown by cell 2 in Figure 6B. No nuclei classified as low Ftz expression state at gastrulation were predicted to express high Ftz after gastrulation. Thus, the autoregulatory element ensures that the vast majority of cells adopt a fate matching the transient state at gastrulation.

While it is clear that the autoregulatory element establishes developmental memory by fixing the Ftz expression fate in steady state, we wondered whether this memory was already at play at gastrulation, or whether the early module is principally responsible for setting Ftz state at gastrulation. Our simulations show that a stripe pattern is already evident at gastrulation from the contribution of the early protein alone, ignoring the autoregulatory contribution (Figure 6D). Thus, it appears that the anterior boundary of stripe 4 at gastrulation is defined by the regulatory activity of upstream factors binding the early element in a manner that is largely independent of the activity of the autoregulatory element. Therefore, this result supports the conclusion that the autoregulatory module is bistable and that it primarily serves to commit cells to fates predetermined by the early element.

2.5 Ftz fate is robustly specified in half an hour

Given that the ftz autoregulatory element acts as a memory module, we might ask how long it takes nuclei to convert the transient expression state of the early module into a stable cellular memory, thereby establishing an expression fate at long times beyond gastrulation. We posit that this timespan corresponds to the classical notion of a commitment window, defined as the period of time during which a cell integrates information from external factors to decide its fate (Dalton, 2015; McNeely and Dwyer, 2021). It can be difficult to access temporal features of development such as the commitment window in vivo, in part due to the technical challenge of measuring and systematically manipulating input signals while simultaneously monitoring the resulting gene expression programs in individual cells within intact tissues or organisms (Bending et al., 2018; Johnson and Toettcher, 2018).

Our mathematical model provided us with a unique opportunity to examine the commitment window by altering the timing of developmental events in silico. Because the low Ftz fate at steady state is the default expression state—the autoregulatory module will always produce zero protein in the absence of a transient signal from the early module—the commitment window primarily determines whether the cell has enough time to detect if transient Ftz concentrations are high and, if so, to adopt a trajectory destined for a high steady-state expression fate. Thus, for the results reported in this section, we restricted our analysis to a subset of the best predicted nuclei, the nuclei that were correctly classified at gastrulation and have relatively low cumulative error between simulation and experiment over time (Section S3.1), that were also predicted to adopt the high fate according to the switching separatrix analysis (N = 21; Figure 6B).

We define the commitment window as \( t_{\text{off}} - t_{\text{on}} \), where \( t_{\text{on}} \) indicates the start of autoregulatory responsiveness and \( t_{\text{off}} \) is the time when upstream factors stop controlling the transcriptional dynamics of the early element (Figure 7A and B). In other words, the commitment window represents the total amount of time during which both the early and autoregulatory modules dictate ftz expression (Figure 7C), and serves as an estimate for how long the autoregulatory module has to establish a memory of transient Ftz state. We define \( t_{\text{off}} \) such that \( r(t) \geq r(t_{\text{off}}) = 0 \), where \( r(t) \) is the mRNA production rate of the early element. Before \( t_{\text{off}} \), \( r(t) \) follows the usual exponential decay with rate \( 1/\beta = 21 \text{ min} \) (Figure 7B).

To determine how long of a commitment window allows cells to convert transiently high Ftz concentrations into permanently high Ftz fates, we asked whether our analyzed subset of nuclei still reached the high fate as we systematically varied the commitment window. We solved the
Figure 7. *In silico* analysis reveals the window of commitment of cells to the high Ftz fate. (A-B) Schematic illustrating (A) $t_{on}$, the time the autoregulatory element becomes responsive, and (B) $t_{off}$, the time the early element ceases production. The commitment window $t_{off} - t_{on}$ is the timespan during which both upstream factors and autoactivation dictate Ftz expression, and serves as an estimate for how long the autoregulatory module has to establish a memory of the signal from the early module. (C) Simulated Ftz concentrations resulting from the timing pictured in A and B. (D) Contour plot showing what percentage of the subset of analyzed nuclei (see text) reach high Ftz fate under wild-type conditions ($N = 21$; Figure 6B, Section S3.1) still reach that fate as the commitment window $t_{off} - t_{on}$ is varied and $t_{on}$ is delayed relative to the measured -20 min (see text). (E) Simulated single-nucleus traces of total Ftz for varying values of $t_{on}$ and the commitment window can cause the transient dynamics to vary quite dramatically. Dashed lines denote the $t_{off}$ (gold) and $t_{on}$ (gray) values used for each plot. Parameters are as in Table S1. See also Figure S10.
dynamical system from Eq. 4 and Eq. 5 with commitment windows of increasing length and start times $t_{on}$ between -20 min and 0 min and recorded which nuclei reached the high Ftz fate. Our results are reported in terms of the fraction of nuclei within the subset that adopted the high Ftz fate for each timing condition.

Figure 7D shows the fraction of nuclei predicted to adopt the high Ftz fate as a function of the commitment window and $t_{on}$. For example, assuming $t_{on} \leq -13$ min, a commitment window of 34 min results in 95% of nuclei achieving high steady-state Ftz levels without further signaling from the early element (Figure 7D). Our results also reveal that there is a gradual dropoff in the fraction of cells that do not commit to the high fate as the commitment window is shortened or $t_{on}$ is delayed. This suggests that slight temporal perturbations during development are unlikely to cause catastrophic patterning failures in which all cells suddenly adopt the low Ftz fate. Rather, we might expect small changes in timing to affect the fates of only a small percentage of cells.

Our analysis of the commitment window indicates that about half an hour can suffice for the vast majority of high-fated cells to stably commit to that fate. Proper fate specification, however, does not guarantee similarity in the temporal trajectories of Ftz concentration, as evidenced by the wide range of dynamics observed in simulated traces for varying $t_{on}$ and $t_{off}$ with the same initial conditions for the early module (Figure 7E; Figure S10). Thus, if the transient Ftz trajectory, not just its ultimate fate, is instructive for downstream genes, then the need for proper regulation of these genes may place stricter constraints on the relative timing of the early and autoregulatory elements than those that are imposed by the specification of steady state Ftz fate alone.

3 Discussion

For decades, developmental biologists have used the concept of Waddington’s landscape to conceptualize the adoption of discrete cell fates. Under this framework, cells roll down valleys in a predetermined landscape to adopt their ultimate fates. This framework has been repeatedly mathematized using dynamical systems theory (Wang et al., 2011; Furusawa and Kaneko, 2012; Jaeger and Monk, 2014; Corson and Siggia, 2017; Sáez et al., 2022). Many of these studies have hypothesized that positive autoregulation (Crews and Pearson, 2009) helps establish and maintain binary cell fates through bistability (Zernicka-Goetz et al., 2009; Soldatov et al., 2019), which can be thought of as introducing forks in Waddington’s landscape. Though experiments in cell culture and fixed tissue have provided evidence for the bistability of various autoregulatory modules found within gene regulatory networks, until now, these results have not been confirmed by direct examination of dynamics in intact, living embryos.

In this work, we utilized live imaging approaches to quantitatively characterize the dynamics of the fruit fly ftz regulatory system in vivo. We elucidated tight temporal coordination between the two enhancer elements that regulate ftz expression (Figure 3), and combined dynamical systems modeling with biophysical measurements to show that the bistability of the autoregulatory module can remember otherwise transient expression levels driven by upstream factors (Figure 6). Based on the prevalence of autoregulatory motifs found in nature (Alon, 2006; Peter and Davidson, 2015), we speculate that the approach employed by the Ftz system to decide cell fate is not limited to fruit flies, but might also be widely adopted during development in other organisms.

One of our central discoveries is that ftz autoregulation is triggered at a specific developmental time rather than by a threshold concentration of Ftz protein driven by the early element. Recent work has suggested candidates for “timer genes” that are expressed at distinct developmental time points and appear to facilitate the expression of other genes (Clark and Akam, 2016; Clark and Peel, 2018; Clark et al., 2022). At least one of these timer genes, Odd-paired, has been shown to exhibit chromatin opening activity (Soluri et al., 2020), and two, Caudal and Dichaete, have been shown to bind directly to the eve autoregulatory element (MacArthur et al., 2009). We speculate that timer
genes might also bind the ftz autoregulatory element to trigger its responsiveness to Ftz protein. Furthermore, the tight temporal coordination we observed between the transcriptional activity driven by the early and autoregulatory elements suggests that sequentially expressed timer genes might differentially regulate enhancers to precisely coordinate gene expression during development.

A basic assumption of our work is that genetic networks exhibit modularity (Hartwell et al., 1999; Bolouri and Davidson, 2002; Del Vecchio et al., 2016), meaning the network can be broken down into parts (modules), each with some inputs and outputs connected to other modules. The behavior of the whole network can then be predicted from the behavior of the modules in isolation. How to define the modules, or equivalently how to break the network down into parts, has been the subject of much discussion (Hartwell et al., 1999). As we have argued in this work, topology, the pattern of interactions among molecular species, is not enough to determine the behavior of a module. Indeed, its dynamics will depend on the values of the parameter governing these interactions (Angeli et al., 2004; Ingram et al., 2006; Graham et al., 2010; Blanchini and Franco, 2014; Khammash, 2016; Verd et al., 2019).

There is inherent flexibility in deciding which physical components to include in a module, and hence which quantities act as parameters and which as inputs to the module. For example, for the ftz autoregulatory system, the distinction between the early and autoregulatory elements led us to structure the autoregulatory module with input $P_{\text{early}}$ and output $P_{\text{late}}$. Figure S2. This is not the only way to define the module; notably, if we knew which regulatory factors rendered the autoregulatory element responsive, then we could include those as inputs and modify the model accordingly.

Our fairly simple representation of the autoregulatory module can predict the fate of ftz expression from arbitrary trajectories of early Ftz. As a result, we can predict the effect of modifications to upstream signaling on the resulting gene expression patterns. This, in turn, allows us to ask what forms of input are appropriate to achieve particular patterning outcomes. Such ability to reverse engineer the process of cellular decision-making could facilitate designing perturbations to manipulate the system, identifying constraints placed on upstream modules by the needs of downstream modules, and analyzing whether biologically evolved signals match those that are mathematically “optimal” for such needs as speed of patterning (Pezzotta and Briscoe, 2022) or information transmission (Tkačik and Gregor, 2021). Different methods of generating predictions may be appropriate depending on the types of inputs under consideration. In this paper, the fact that increasing any one of the parameters that define the early Ftz input ($r_0$, $R_t$, $P_t$) increases total Ftz concentration at all points in time (a property known as monotonicity; Angeli et al. (2004); Sootla et al. (2016)) made it possible to analyze our model using a switching separatrix. However, this may not be true for other regulatory systems, as in the case where a gene within a module represses its own production.

Throughout developmental biology, the concept of a commitment window has been repeatedly utilized to describe the amount of time cells need to be exposed to upstream signals in order to decide their developmental fates (Dalton, 2015; McNeely and Dwyer, 2021). Our quantitative dynamical systems model made it possible to conduct a detailed examination of this commitment window, and to identify what fraction of cells adopt certain fates as developmental timing is varied. From an engineering perspective, we may consider a gene expression pattern as a “design specification” that must be achieved with a prescribed level of precision, and work backwards to see what inputs satisfy this requirement. Our approach complements existing work on precision that emphasizes how tightly protein concentrations are controlled (Gregor et al., 2007) and how accurately cells can locate their position by reading out concentrations of upstream factors (Dubuis et al., 2013; Petkova et al., 2019). In particular, the latter approaches indicate what level of precision is actually
achieved by a patterning network, while our framing focuses rather on what range of parameters allow a system to attain a predefined level of precision. A combination of the two perspectives could help elucidate what biophysical and evolutionary factors influence stochastic variation in phenotypes, including how precise expression patterns must actually be to produce functional, healthy organisms. In any case, we hope our results act as one more step toward grounding robustness or reproducibility in developmental patterning on a quantitative, probabilistic level.

In summary, by turning widespread schematic models of autoactivation modules into precise mathematical statements and experimentally testing the resulting predictions, we have provided support for a widely held hypothesis about how developmental fates are established in embryos. In the future, combining quantitative measurements with precise spatiotemporal perturbations (Goglia and Toettcher, 2019) and synthetic reconstitution methods (McNamara et al., 2022) promises to enable yet another iteration of the dialogue between theory and experiment that constitutes the basis of our work, ultimately leading to a predictive understanding of function in developmental networks and the myriad forms and fates to which they give rise.
Methods and Materials

Cloning and Transgenesis

The fly lines used in this study were generated by inserting transgenic reporters into the fly genome or by CRISPR-Cas9 genome editing, as described below. See Table S2 for detailed information on the plasmid sequences used in this study.

Creation of tagged *fushi tarazu* (*ftz*) gene using CRISPR-Cas9

*Ftz-EGFP-LlamaTag* fusion design is based on previously published transgenic line (*Bothma et al., 2018*). To tag endogenous *ftz* locus with EGFP-LlamaTag, we used CRISPR-mediated homology-directed repair with donor plasmid synthesized by Genscript. gRNA was designed using the target finder tool from flyCRISPR (https://flycrispr.org), and cloned based on the protocol from *Gratz et al. (2015)*. *yw;* nos-Cas9(II-attP40) transgenic line was used as the genomic source for Cas9 and the embryos were injected and screened by BestGene Inc.

Creation of *ftz* autoregulatory element reporter

The *ftz* autoregulatory element sequence is based on 4.4kb DNA segment described in (*Hiromi et al., 1985*). The *ftz* autoregulatory element reporter was constructed by combining the enhancer sequence with an array of 24 MS2 stem loops fused to the *D. melanogaster yellow* gene (*Bothma et al., 2018*). The construct was synthesized by Genscript and injected by BestGene Inc into *D. melanogaster* embryos with a ΦC31 insertion site in chromosome 3 (Bloomington stock #9750; landing site VK00033; cytological location 65B2).

Transgenes expressing EGFP and MCP-mCherry

The fly line maternally expressing MCP-mCherry (chromosome 3) was constructed as described in (*Bothma et al., 2018*). The fly line maternally expressing vasa-EGFP (chromosome 2) was constructed as described in (*Kim et al., 2021*). To simultaneously image protein dynamics using LlamaTags and transcription using MCP-MS2 system, we combined the vasa-EGFP transgene with MCP-mCherry to construct a new line (*yw;* vasa-EGFP;CyO; MCP-mCherry,FtzAuto-MS2-Yellow, Ftz-LlamaTag) that maternally expresses both proteins.

Fly lines

To measure Ftz transcription and protein levels simultaneously, we performed crosses to generate virgins carrying transgenes that drive maternal EGFP, MCP-mCherry, the LlamaTagged Ftz locus along with *ftz* autoregulatory element reporter (*yw;* vasa-EGFP,CyO,FtzAuto-MS2-Yellow, Ftz-LlamaTag). These flies were then crossed with males having both the *ftz* autoregulatory element reporter and the LlamaTagged Ftz locus (*yw;* +; FtzAuto-MS2-Yellow, Ftz-LlamaTag). This resulted in the embryo carrying maternally deposited EGFP, MCP-mCherry, and two copies of the LlamaTagged Ftz locus and *ftz* autoregulatory element reporter.

Embryo preparation and data collection

The embryos were prepared following procedures described in (*Garcia et al., 2013; Bothma et al., 2018; Lammers et al., 2020*). Embryos were collected and mounted in halocarbon oil 27 between a semipermeable membrane (Lumox film, Starstedt, Germany) and a coverslip. Confocal imaging on a Zeiss LSM 780 microscope was performed using a Plan-Apochromat 40x/1.4NA oil immersion objective. EGFP and MCP-mCherry were excited with laser wavelengths of 488 nm (25.0 μW laser power) and 594 nm (15.0 μW laser power), respectively. Fluorescence was detected using the Zeiss QUASAR detection unit. Image resolution was 512 x 512 pixels, with pixel size of 0.231 μm. Sequential z-stacks separated by 0.5 μm were acquired. Specimens were imaged from mid nuclear cycle 14 until the start of gastrulation.
Image processing

Image analysis of live embryo movies was performed based on the protocol in Garcia et al., 2013; Reimer et al., 2021, which included nuclear segmentation, spot segmentation, and tracking. In addition, the nuclear fluorescence of Ftz was calculated based on a nuclear mask generated from the MCP-mCherry channel. Ftz concentration for individual nuclei was extracted based on the integrated amount from maximum projection along the z-stack. The GFP background was calculated based on a control experiment and subsequently subtracted from the data.

Numerical analysis and simulations

Numerical analysis and simulations were carried out using custom scripts in MATLAB (2017b). The switching separatrix and analogous surface for transient Ftz state at gastrulation were estimated using a modification of the algorithm in Sootla et al., 2016, which employs a combination of bisection and random sampling to estimate the upper and lower bounds of the separatrix surface. Stochastic differential equations were simulated using the Euler-Maruyama method. More detailed descriptions of the procedures are available in the Supplementary Text.

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Author contributions

Conceptualization: JZ, MLP, HGG
Methodology: JZ, MLP, HGG
Resources: JZ, MLP, MN, HGG
Investigation: JZ, MLP, HGG
Visualization: JZ, MLP, HGG
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Supervision: HGG
Writing – original draft: JZ, MLP, HGG
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Declaration of interests

The authors declare no competing interests.

Data and materials availability

All materials are available upon request. All data in the main text or supplementary materials are available upon request. All code is available in this paper’s github repository.
Supplementary Information

Supplementary Figures

Figure S1. Single-cell traces of Ftz protein and autoregulatory activity show that high or low Ftz concentration results in distinct temporal dynamics. Green and magenta lines correspond to Ftz protein and transcriptional activity of the autoregulatory element, respectively, obtained as described in fig:autoregulationA. (A) High level of Ftz protein results in continued active transcription from the autoregulatory element. (B) Low level of Ftz protein results in a much weaker autoregulatory response.
Figure S2. Two ways to visualize a genetic network as an interconnection of modules. (A) Schematic of Ftz regulation in which populations of Ftz protein are differentially labeled based on whether they are generated by regulation from the early element ($P_{\text{early}}(t)$) or from the autoregulatory element ($P_{\text{late}}(t)$). The two populations are summed to generate the total Ftz concentration that binds the autoregulatory element. (B) The Ftz regulatory system can be visualized as a block diagram to highlight how a modular network decomposition might be inspired by similar breakdowns of electrical circuits and other engineering control systems (Cosentino and Bates, 2011). Here, unspecified upstream factors regulate the early element module, which produces output $P_{\text{early}}(t)$. This, in turn, acts as the input to the autoregulatory module, which has been schematized as a latch with a clock signal (clk) to show that the element only becomes responsive at a particular time. The output $P_{\text{late}}(t)$ can be summed with $P_{\text{early}}(t)$ to recover total Ftz concentration. Relative to the more traditional schematic in (A), this representation includes dynamic signals ($P_{\text{early}}(t)$ and $P_{\text{late}}(t)$), a timing element that triggers autoregulatory responsiveness, and a clear separation of inputs and outputs from each module.
### Supplementary Tables

#### Table S1. Quantitative parameters used throughout this work

| Parameter | Description | Source | Value         | Units                          |
|-----------|-------------|--------|---------------|-------------------------------|
| $\alpha$  | translation rate | this work | 0.082 $\pm$ 0.004 | protein AU (mRNA AU min)$^{-1}$ |
| $\tau_R$  | mRNA half-life | (Edgar et al., 1986) | 7 | min |
| $\gamma_R$ | mRNA decay rate | $\frac{R}{\tau_R}$ | 0.0990 | min$^{-1}$ |
| $\tau_P$  | protein half-life | (Bothma et al., 2018) | 7.9 | min |
| $\gamma_P$ | protein decay rate | $\ln 2 \cdot \frac{R}{\tau_P}$ | 0.0877 | min$^{-1}$ |
| $f(P)$    | autoactivation gene regulatory function | this work | $\frac{\alpha P}{K + P}$ | mRNA AU (protein AU)$^{-1}$ |
| $a$       | maximum transcription rate | this work | 5.555e5 $\times$ 10$^5$ | mRNA AU (protein AU)$^{-1}$ |
| $K$       | -  | this work | 1.216$\times$10$^6$ | protein AU |
| $n$       | Hill coefficient | this work | 3.264 | (none) |
| $c$       | ratio of transcription rate endogenous to transgene | this work | 0.45 $\pm$ 0.02 | (none) |
| $\beta$   | decay rate of early mRNA production | this work | 0.0479 $\pm$ 0.0021 | min$^{-1}$ |

#### Table S2. List of plasmids used in this study.

| Name                          | Function                                                   |
|-------------------------------|------------------------------------------------------------|
| pBPhi-FtzEarly-MS2            | ftz early element MS2 reporter                             |
| pBPhi-FtzUpstream-MS2-Yellow  | ftz autoregulatory element MS2 reporter                    |
| pUC57-Ftz-LlamaTag-dsRed      | Donor plasmid for Ftz-LlamaTag CRISPR knock-in fusion     |
| pU6-3-gRNA-Ftz-1              | guide RNA 1 for Ftz-LlamaTag CRISPR knock-in fusion        |
| pU6-3-gRNA-Ftz-2              | guide RNA 2 for Ftz-LlamaTag CRISPR knock-in fusion        |
| pU6-3-gRNA-Ftz-3              | guide RNA 3 for Ftz-LlamaTag CRISPR knock-in fusion        |

**Supplementary Movie**

**Movie S1.** Two-color imaging of input Ftz protein concentration and the output autoregulatory dynamics at stripes 3, 4 and 5.
Supplementary Text

S1 Modeling

S1.1 Autoregulatory element

We use $R_{\text{early}}(t)$ to describe the concentration of ftz mRNA transcribed from the early element, which is translated into protein $P_{\text{early}}(t)$. We define $R_{\text{late}}(t)$ as the ftz mRNA transcribed from the autoregulatory element and translated into protein $P_{\text{late}}(t)$. The total Ftz protein in the cell at time $t$ is given by $P_{\text{total}}(t) = P_{\text{early}}(t) + P_{\text{late}}(t)$. The dynamical equations describing the temporal evolution of mRNA and protein are

\[
\text{autoregulatory element: } \begin{cases} 
\frac{dR_{\text{late}}}{dt} = c \hat{f}(t, P_{\text{total}}(t)) - \gamma_R R_{\text{late}}(t) \\
\frac{dP_{\text{late}}}{dt} = \alpha R_{\text{late}}(t) - \gamma_P P_{\text{late}}(t)
\end{cases} \tag{S1}
\]

where $\gamma_R$ and $\gamma_P$ are the decay rates of mRNA and protein respectively, $\alpha$ is the translation rate, and $c$ is a scaling factor equivalent to the ratio of maximum production rate from the endogenous locus vs. the transgene (where $\hat{f}$ is measured). Note that $\hat{f}(t, \cdot)$, the gene regulatory function for the autoregulatory element, is time dependent. Unless otherwise stated, we will assume

\[
\hat{f}(t, P) = \begin{cases} 
0, & t < t_{\text{on}} \\
f(P), & t \geq t_{\text{on}}
\end{cases} \tag{S2}
\]

where

\[
f(P) = \frac{aP^n}{K^n + P^n} + l \tag{S3}
\]

is the sigmoid describing the autoregulatory relationship at maximum amplitude. Using the definition of $P_{\text{total}}(t)$ we will then write Eq. S1 as

\[
\text{autoregulatory element: } \begin{cases} 
\frac{dR_{\text{late}}}{dt} = c \hat{f}(P_{\text{early}}(t) + P_{\text{late}}(t)) - \gamma_R R_{\text{late}}(t) \\
\frac{dP_{\text{late}}}{dt} = \alpha R_{\text{late}}(t) - \gamma_P P_{\text{late}}(t)
\end{cases}, t \geq t_{\text{on}}. \tag{S4}
\]

Thus, $P_{\text{early}}(t)$ acts as the sole time-varying input to the autoregulatory element. Since autoregulation does not begin until time $t_{\text{on}}$, the initial conditions for Eq. S4 are fixed at $R_{\text{late}}(t_{\text{on}}) = 0$ and $P_{\text{late}}(t_{\text{on}}) = 0$.

S1.2 Early element

From our empirical measurements, we observed that the production rate $r(t)$ of early ftz mRNA is well approximated by an exponential decay (Figure S4), allowing us to model $R_{\text{early}}(t)$ and $P_{\text{early}}(t)$ through the dynamical system

\[
\begin{align*}
\frac{dr}{dt} &= -\beta r(t) \\
\frac{dR_{\text{early}}}{dt} &= r(t) - \gamma_R R_{\text{early}}(t) \\
\frac{dP_{\text{early}}}{dt} &= \alpha R_{\text{early}}(t) - \gamma_P P_{\text{early}}(t)
\end{align*} \tag{S5}
\]

As before, $\alpha$ is the translation rate and $\gamma_R$ and $\gamma_P$ are the decay rates of mRNA and protein.

Because Eq. S5 is linear, it can be equivalently written as

\[
\frac{d\vec{x}(t)}{dt} = \begin{bmatrix} -\beta & 0 & 0 \\ 1 & -\gamma_R & 0 \\ 0 & \alpha & -\gamma_P \end{bmatrix} \vec{x}(t) =: A\vec{x}(t), \tag{S6}
\]
where

\[ \bar{\chi}(t) = \begin{bmatrix} r(t) \\ R_{\text{early}}(t) \\ P_{\text{early}}(t) \end{bmatrix} \]  \hspace{1cm} (S7)

The analytical solution is then given by

\[ \bar{\chi}(t) = c_1 e^{\lambda_1 t} \bar{v}_1 + c_2 e^{\lambda_2 t} \bar{v}_2 + c_3 e^{\lambda_3 t} \bar{v}_3, \]  \hspace{1cm} (S8)

where \( \lambda_1, \lambda_2, \lambda_3 \) are the eigenvalues of \( A \) corresponding to eigenvectors \( \bar{v}_1, \bar{v}_2, \bar{v}_3 \) respectively. In particular,

\[ \bar{\chi}(t) = \begin{bmatrix} 1 \\ \frac{1}{\gamma_R - \beta} \\ \frac{\gamma_R - \beta}{\lambda_1} \end{bmatrix} e^{-\gamma_R t} + \begin{bmatrix} 0 \\ 1 \\ 0 \end{bmatrix} e^{-\gamma_P t} + \begin{bmatrix} 0 \\ 0 \\ 1 \end{bmatrix}, \]  \hspace{1cm} (S9)

where

\[ c_1 = r(t_m), \ c_2 = \left( R_{\text{early}}(t_m) - \frac{r(t_m)}{\gamma_R - \beta} \right), \ c_3 = P_{\text{early}}(t_m) - \frac{\alpha}{\gamma_P - \gamma_R} c_2 - \frac{\alpha}{(\gamma_R - \beta)(\gamma_P - \beta)} c_1. \]  \hspace{1cm} (S10)

Thus, the input \( P_{\text{early}}(t) \) to the autoregulatory element is completely characterized by three parameters \( (r(t_m), R_{\text{early}}(t_m), P_{\text{early}}(t_m)) \) corresponding to the initial conditions for Eq. S5. Since every term in the solution is multiplied by an exponential that decays in time, all state variables will tend to 0 as \( t \) goes to infinity.

S1.3 Intersection test for bistability

In Box 1 in the main text we give an example of how to identify stable steady states in a model that only acknowledges protein concentration and ignores mRNA dynamics. Here, we show how to use the graphical intersection test on systems including both mRNA and protein.

To reiterate, our dynamical system is described by Eq. S4 is given by

\[
\begin{align*}
\frac{dR_{\text{late}}}{dt} &= c_f (P_{\text{early}}(t) + P_{\text{late}}(t)) - \gamma_R R_{\text{late}}(t) \\
\frac{dP_{\text{late}}}{dt} &= \alpha R_{\text{late}}(t) - \gamma_P P_{\text{late}}(t)
\end{align*}
\]  \hspace{1cm} (S11)

Steady states are system states at infinite time. Since we know that \( P_{\text{early}}(t) \) goes to 0 at long times, the steady states for Eq. S4 are equivalent to the steady states of

\[
\begin{align*}
\frac{dR_{\text{late}}}{dt} &= c_f (P_{\text{late}}(t)) - \gamma_R R_{\text{late}}(t) \\
\frac{dP_{\text{late}}}{dt} &= \alpha R_{\text{late}}(t) - \gamma_P P_{\text{late}}(t)
\end{align*}
\]  \hspace{1cm} (S12)

which has no input and can therefore be analyzed for steady states by standard methods.

By definition, at steady state the derivative of the different molecules species with respect to time equals zero, allowing us to write

\[
\begin{align*}
\frac{dR_{\text{late}}}{dt} &= 0 = c_f (P_{\text{late}}) - \gamma_R R_{\text{late}} \\
\frac{dP_{\text{late}}}{dt} &= 0 = \alpha R_{\text{late}} - \gamma_P P_{\text{late}}
\end{align*}
\]  \hspace{1cm} (S13)

where \( (R_{\text{late}}^*, P_{\text{late}}^*) \) is a steady state. Then, we rearrange the bottom equation to get

\[ R_{\text{late}}^* = \frac{\gamma_P}{\alpha} P_{\text{late}}^*, \]  \hspace{1cm} (S14)

which we plug into the top equation to get

\[ 0 = c_f (P_{\text{late}}^*) - \frac{\gamma_P f_P}{\alpha} P_{\text{late}}^*. \]  \hspace{1cm} (S15)
From here, we rearrange terms to recover

$$\gamma_P P^*_{\text{late}} = \frac{ac}{f_R} f(P^*_{\text{late}}).$$  \hfill (S16)

Hence, the intersections of a line of slope $\gamma_P$ with the right-hand side give the steady-state late protein concentrations $P^*_{\text{late}}$, from which we can recover $R^*_{\text{late}}$ through Eq. S14. For a plot of the intersection test, see Figure 6A in the main text.

**S2 Parameter estimation**

**S2.1 Regulatory function $f(P)$ of the ftz autoregulatory element**

We calculated the regulatory function ($f(P)$) of the ftz autoregulatory element (shown in Figure 4D) for the anterior boundary of stripe 4. To make this possible, we identified the boundary in a manually selected image frame prior to gastrulation by extracting two adjacent columns of cells, each corresponding to high or low Ftz concentration. For each cell, we obtained the MS2 signal, which is a proxy for the instantaneous rate of transcription (Garcia et al., 2013; Bothma et al., 2014; Lamers et al., 2020), and the Ftz fluorescence for each time point. Next, we binned data points within a specific temporal window into ten quantiles. We averaged the MS2 and Ftz signals belonging to the same quantile, then fit a Hill function to the resulting values to obtain the regulatory function of the ftz autoregulatory element within that time window. We repeated the process in $N = 7$ embryos and pooled data from all embryos for subsequent analysis over four temporal windows (-20 to -15, -15 to -10, -10 to -5, and -5 to 0 min) to obtain the trend shown in Figure 4E.

**S2.2 Translation rate $\alpha$**

To calculate the translation rate, we simultaneously imaged the ftz transcription rate and the resulting Ftz protein concentration in a Ftz-MS2-LlamaTag construct (Figure S3A). We focused on the nuclei with no initial Ftz transcription. For these nuclei, we measured the MS2 signal (see Figure S3B for a sample trace), which is an approximation of the ftz mRNA production rate (Garcia et al., 2013; Lamers et al., 2020; Bothma et al., 2018), and integrated this signal in order to obtain the total amount of mRNA produced (see Figure S3C; Garcia et al. (2013)). This integration was done by solving the differential equation for the mRNA $R(t)$ given by

$$\frac{dR}{dt} = r(t) - \gamma_R R(t),$$  \hfill (S17)

where $r(t)$ is the transcription rate (i.e., the mRNA production rate reported by MS2 fluorescence), and $\gamma_R = 0.099 \text{ min}^{-1}$ (see Table S1). An example of a resulting prediction for the amount of mRNA as a function of time is shown in Figure S3C.

Next, we performed a parameter sweep for the translation rate $\alpha$ and, for each value of $\alpha$, we integrated $R(t)$ to predict the protein dynamics $P(t)$ using the following equation (see Figure S3D for sample nuclear traces)

$$\frac{dP}{dt} = \alpha R(t) - \gamma_P P(t).$$  \hfill (S18)

The translation rate that results in the best fit (Figure S3D, red line) is recorded for each nucleus. We then calculated $\alpha$ values for each embryo by averaging best-fitted $\alpha$ for each single cell (Figure S3E). These values are averaged across $N = 129$ cells (embryo 1) and $N = 119$ cells (embryo 2), respectively. Then we averaged the resulting value of $\alpha$ between two embryos, giving us $\alpha = 0.082 \pm 0.004$ protein AU (mRNA AU min$^{-1}$) (see Table S1), which is used in our dynamical systems model in the main text.
Figure S3. Procedure for estimating the translation rate $\alpha$. (A) Ftz-MS2-LlamaTag transgenic construct used for estimating the translation rate. (B) Illustrative example of the raw MS2 traces that report on the instantaneous ftz transcription rate. MS2 traces are smoothed using a moving average of 1 min. (C) We integrate the MS2 traces to predict the mRNA dynamics in individual nuclei. (D) For a given value of the translation rate, we integrate the predicted mRNA dynamics to subsequently predict the Ftz protein dynamics in individual nuclei. The value of $\alpha$ that leads to the best agreement between prediction and experiment is found (red line). Suboptimal fits are shown as (gray lines). Ftz protein traces are smoothed using a moving average of 5 min. (E) Histogram distribution of best-fitted $\alpha$ values for individual cells within two embryos. Note that $\alpha$ values larger than 0.2 au are omitted for accuracy ($N = 2$ nuclei). We first calculated the average $\alpha$ for each individual embryo and then computed the average between these two embryos, resulting in $\alpha = 0.082 \pm 0.004$ protein AU (mRNA AU min)$^{-1}$, which is used in our dynamical systems model.
**S2.3 Early element transcriptional activity decay rate $\beta$**

We calculated the decay in the mRNA production rate of early element, described by the decay rate $\beta$, from fluorescence measurements of the early element MS2 reporter construct (Figure S4A). We performed an exponential fit to the average trajectory of each embryo (see Figure S4B for an example fit) and averaged the resulting decay rates to obtain the mean value $\beta = 0.048 \pm 0.0021$ (see Table S1, $N = 2$ embryos) that we used in the dynamical systems model.

**S2.4 Scaling factor $c$**

We estimated $c$, the ratio of maximum production rate from the endogenous locus vs. the transgene, by approximating the solution to the dynamical system in Eq. S4 between two time points for which we have empirical data. In particular, we began from the experimental observation that $\tilde{f}(t, P)$ plateaus shortly before gastrulation for nuclei with total Ftz levels above about $P_{\text{thresh}} = 1.5 \times 10^6$ a.u. Therefore, for nuclei that satisfy $P_{\text{early}}(t) \geq P_{\text{thresh}}$ during this time (Figure S5A), we can approximate the nonlinear system for the autoregulatory element by the following linear system

$$
\begin{align*}
\frac{dR}{dt} &= c f(P_{\text{thresh}}) - \gamma_R R(t), \\
\frac{dP}{dt} &= \alpha R(t) - \gamma_P P(t).
\end{align*}
$$

(S19)
where \( f(P_{\text{ onCreate}}) \) is now a constant. This system of equations has an analytical solution given by

\[
\begin{align*}
R(t) &= c R(0) e^{-\gamma t} + \frac{c f(P_{\text{ onCreate}})}{\gamma} \left( 1 - e^{-\gamma t} \right) \\
P(t) &= P(0) e^{-\gamma t} + \frac{\alpha}{\gamma P - \gamma R} \left( R(t) - R(0) e^{-\gamma t} - \frac{c f(P_{\text{ onCreate}})}{\gamma} \left( 1 - e^{-\gamma t} \right) \right).
\end{align*}
\]

(S20)

Rearranging these expressions gives

\[
c = \frac{\gamma P}{f(P_{\text{ onCreate}})} \left( \left( R(t) - R(0) e^{-\gamma t} \right) - \frac{\gamma P - \gamma R}{\alpha} \left( P(t) - P(0) e^{-\gamma t} \right) \right).
\]

(S21)

Note that here \( t = 0 \) is assigned to the beginning of the time window over which the simulation is performed.

We calculated the solution to Eq. S21 for the individual boundary nuclei in the same embryos as used to fit the gene regulatory function (Figure 4D). We restricted our estimations to the 3 min before gastrulation based on personal observations that the prediction accuracy of the simulation of this linearized system tended to fall after ~3 min. We derived the initial conditions \((R(0), P(0))\) from estimates of the late protein obtained by subtracting simulated early protein from the total protein trace (where the autoregulatory element was assumed to begin contributing at -20 min before gastrulation). We estimated \( \varepsilon \) in a windowed approach whereby, for each nucleus, we simulated only over one empirical sample interval (10 s) for all intervals from 3 min before gastrulation (Figure S5B). We pooled all samples for individual time windows across all nuclei (Figure S5C and D) and averaged them to give a final estimate of \( \varepsilon \approx 0.45 \pm 0.02 \) across the 3 min before gastrulation (where the error range is the standard error).

**S3 Simulations**

**S3.1 Best predicted nuclei**

In order to be assured of the accuracy of our conclusions concerning the dynamics of the commitment process, in Figure 7 we decided to restrict our analysis to sets of nuclei whose simulated trajectories well matched the empirical traces. From our dataset, we identified such “best predicted” nuclei based on the cumulative error between a measured trajectory \( \{(R_i, P_i)\} \) at discrete time points \( t \) and a simulated trajectory \( \{(\hat{R}_i, \hat{P}_i)\} \) at the same time points as

\[
\varepsilon = \sum_t \sqrt{(R_t - \hat{R}_t)^2 + (P_t - \hat{P}_t)^2}.
\]

(S22)

From a histogram of the errors (Figure S6A), which was roughly bimodal, we identified a threshold of \( 7 \times 10^5 \) to identify the 79 best predicted nuclei out of 118 nuclei total. Some sample traces from these best predicted nuclei are shown in Figure S6B while traces for nuclei with high cumulative error are shown in Figure S6C.

We also calculated the cumulative error when simulations were conducted for a gradual increase in autoregulatory responsiveness described by

\[
\hat{f}(t, P) = 1 - e^{-\xi(t - t_{\text{ on}})} f(P), \quad t > t_{\text{ on}},
\]

(S23)

where \( f(P) \) is as defined in Eq. S3 and \( \xi = 0.14 \text{ min}^{-1} \) corresponds to a half-life of 5 min (from the observation that the autoregulatory element transitions from unresponsive to fully responsive over 10 min; see Figure 4E). The resulting sample traces are shown in Figure S7B. Compared to the case where we assume that the autoregulatory element becomes active instantaneously at \( t_{\text{ on}} \) (Figure S6A), there was a slight shift in the distribution toward lower error (88 best predicted with the same cutoff as before in Figure S6A), but the binary classification accuracy at gastrulation was the same. Therefore we opted to use \( f(P) \) rather than \( \hat{f}(t, P) \) for the main analysis.
Figure S5. Procedure for estimating \( c \), the ratio of the maximum mRNA production rate from the endogenous to the transgene. (A) We use a subset of samples for which the gene regulatory function is approximately saturated across the entire 3 min before gastrulation. Red, the gene regulatory function. Gray dots are individual time points from individual nuclei during the 3 min before gastrulation. Dark gray dots belong to nuclei for which \( P_{\text{total}}(t) > P_{\text{thresh}} \) for all times between -3 and 0 min relative to gastrulation. Light gray dots correspond to nuclei that did not match this criterion. (B) Top, estimated late mRNA traces and bottom, estimated late protein traces for four sample nuclei drawn from the subset identified in (A). Gray dashed lines indicate one sample interval from \( t' \) to \( t' + \Delta \) used to estimate \( c \) (see text). (C) Gray, estimates of \( c \) for each interval pictured in (B) and each nucleus identified in (A). Red, the mean at each time point across all nuclei. Note that most variation is between nuclei rather than across time points. (D) Histogram of \( c \) for all time intervals across all nuclei, mean 0.45, standard error 0.02.
Figure S6. Classifying nuclei by the cumulative error in model prediction in a model of instantaneous autoregulation onset. (A) Histogram of cumulative errors for $N = 118$ nuclei. Simulations were conducted assuming that the autoregulatory element becomes instantaneously responsive, as in Eq. S3 and the main text. (B) Sample traces of best predicted nuclei. In each plot, the blue curve is the measured trace and the black line is the corresponding predicted trace. The dashed gray line is the threshold for classifying a nucleus as “on” at gastrulation. (C) As for (B), but for nuclei that did not qualify to be best predicted.
Figure S7. Classifying nuclei by the cumulative error in model prediction in a model of gradual autoregulation onset. (A) Histogram of cumulative errors for $N = 118$ nuclei. Simulations were conducted assuming a gradual increase in responsiveness of the autoregulatory element, following the dynamics in Eq. S23. (B) Sample traces of best predicted nuclei. In each plot, the blue curve is the measured trace and the black line is the corresponding predicted trace. The dashed gray line is the threshold for classifying a nucleus as “on” at gastrulation. (C) As for (B), but for nuclei that did not qualify to be best predicted.
S3.2 Whole-embryo simulations

We were curious about how accurately our model, which is based on measurements at the anterior boundary of stripe 4, would predict Ftz state for all nuclei spanning stripes 3, 4 and 5 measured during our experiments. Assuming all nuclei follow the same dynamics as given in Eq. 5 and Eq. S4, we repeated the analysis from Figure 5 to predict Ftz concentration at gastrulation. We achieved a binary classification accuracy of (74.6%, or 763 of 1036 nuclei). Interestingly, this is worse than the accuracy achieved from thresholding early protein alone (84.3%, or 873 of 1036 nuclei), which is itself comparably accurate to the predictions for the anterior boundary of stripe 4 (with or without the autoregulatory contribution). The bulk of classification errors for the whole-embryo simulations, whether from thresholding full simulations or thresholding early protein alone, were false positives at the posterior boundaries of stripes, as in the example plotted in Figure S8. This indicates that something differs in the regulation of Ftz at the anterior boundaries of stripes compared to the posterior boundaries. For example, it has been noted that the posterior, but not the anterior, boundaries of Ftz stripes are repressed by sloppy paired (slp) (Clark, 2017).

S3.3 Delaying the onset of responsiveness of the autoregulatory element

From a mathematical standpoint, we can treat a delay in $t_{on}$ as a change in the starting time of the simulation, which introduces a corresponding change to the initial conditions $(r(t_{on}), R_{early}(t_{on}), P_{early}(t_{on}))$ of the early module. In this way, the time at which the trajectory of the early module $(r(t), R_{early}(t), P_{early}(t))$ crosses the switching separatrix is the latest time at which the autoregulatory element can become responsive and still commit a cell to the appropriate (high) fate (Figure S9). This follows from three conditions: (1) the early element is time invariant, (2) the early element is independent of the autoregulatory module (the same is not true for the autoregulatory module, which takes the output of the early module as its input), and (3) Ftz fate corresponds to the Ftz concentration state at infinite time. (1) and (2) ensure that, even if we delay the autoregulatory element, we can continue to use Eq. 5 to simulate early protein by just changing the initial conditions, while (3) ensures that the delayed start of the autoregulatory element will not change the location.
Figure S9. Sensitivity of Ftz fate to the autoregulatory onset time $t_{on}$. Dashed gray lines denote the trajectories $(r(t), R_{early}(t), P_{early}(t))$ corresponding to each nucleus. Trajectories begin at the colored points, each of which corresponds to the state of the early module in a high-fated nucleus ($N = 21$ same as analyzed in Figure 7) at time -20 min. Nuclei are colored based on the amount of time until the trajectory crosses the switching separatrix from Figure 6B, with darker colors indicating longer times. As explained in the text, this crossing time corresponds to the longest acceptable delay in the onset of autoregulatory responsiveness without changing the fate of the cell. Intriguingly, the trajectories of early protein run parallel to the separatrix before converging to cross it in a restricted region of parameter space.

of the switching surface (which is relative to steady state, not to a transient state of the trajectory at a fixed point in time).

In Figure 7 we analyze the commitment window by varying $t_{off}$ at the same time as $t_{on}$. In Figure S10 we report full results for simultaneous variation in $t_{on}$ and $t_{off}$. The strictness with which cell fate must be specified determines the variation in timing that can be tolerated. For example, if the early element ceases production at or after gastrulation ($t_{off} > 0$), the autoregulatory element can delay responsiveness until -15 min and still guide at least 75% of cells to the appropriate fate. If the early element does not cease production until 20 min, then the autoregulatory element may turn on just after -6 min and still direct 75% of cells to the correct fate. From these results, we see that almost no cells commit to the high fate when $t_{on} > t_{off}$. For this reason, in the main text we always set $t_{off} > t_{on}$.

S3.4 Stochastic simulations

Stochasticity in gene expression during embryonic development can compromise or improve system function depending on the context (Zhang et al., 2012; Papadopoulos et al., 2019). We sought to investigate whether stochasticity in gene expression (1) was sufficient to explain the prediction error rates of our deterministic models, and (2) could drive stochastic switching at appreciable
rates. We examined these questions using stochastic differential equations (SDEs), assuming that the noise in our data arises solely from stochastic dynamics within the cells rather than from measurement noise. Generally, we expect this method to overestimate the error.

In a stochastic differential equation model, changes in the amount of late RNA $\frac{dR_{\text{late}}}{dt}$ and total protein $\frac{dP_{\text{total}}}{dt}$ over a time interval $dt$ are given by

$$
\begin{align*}
\frac{dR_{\text{late}}}{dt} &= (c f(P_{\text{total}}(t)) - \gamma R_{\text{late}}(t)) dt + \sigma_R(R_{\text{late}}(t)) dW \\
\frac{dP_{\text{total}}}{dt} &= (a R_{\text{late}}(t) - \gamma_P P_{\text{total}}(t)) dt + \sigma_P(P_{\text{total}}(t)) dW,
\end{align*}
$$

where $dW$ is Gaussian with mean 0 and variance $dt$ (Gillespie, 2007). The terms $\sigma_R(R_{\text{late}})$ and $\sigma_P(P_{\text{total}})$ scale the variance of the noise from one time increment to the next.

For this analysis, we used our experimental setup featuring endogenous Ftz-LlamaTag driving an autoregulatory element transgene tagged with MS2 as introduced in Figure 4. Because, over a small time interval where mRNA degradation is negligible, the MS2 signal reports on the rate of mRNA production, this signal gave us direct access to $\frac{dR_{\text{late}}}{dt}$ at discrete time points $t_i$. As a result, we can use this measure of $\frac{dR_{\text{late}}}{dt}$ to estimate for the late mRNA $R_{\text{late}}(t)$ by integrating the MS2 signal following

$$R_{\text{late}}(t_i) = R_{\text{late}}(t_{i-1}) + \frac{dR_{\text{late}}(t_i)}{dt} (t_i - t_{i-1})$$

under the assumption that $R_{\text{late}}(t_0) = 0$ (meaning that the autoregulatory element only becomes responsive at time $t_0$). Since we also have direct measurements of $P_{\text{total}}(t)$, this allows us to rearrange Eq. S24 so as to estimate the noise contribution $\sigma_R(R_{\text{late}}(t)) dW$ from

$$\sigma_R(R_{\text{late}}(t)) dW = dR_{\text{late}}(t) - (c f(P_{\text{total}}(t)) - \gamma_R R_{\text{late}}(t)) dt.$$  

If we further assume that $R_{\text{early}}(t)$ is noiseless and therefore given by the deterministic solution in Eq. S9, we can estimate the total mRNA as $R_{\text{total}}(t) = R_{\text{early}}(t) + R_{\text{late}}(t)$. Then, since we have simultaneous measurements of total Ftz protein $P_{\text{total}}$, we can also rearrange the lower equation in Eq. S24 to estimate $\sigma_P(P_{\text{total}}(t)) dW$ from

$$\sigma_P(P_{\text{total}}(t)) dW = dP_{\text{total}}(t) - (a R_{\text{total}}(t) - \gamma_P P_{\text{total}}(t)) dt.$$ 

---

**Figure S10. Dependence of Ftz fate adoption on the time that autoregulatory responsiveness begins and production rate from the early element shuts down.** Left, heat map showing the fraction of cells predicted to adopt the high Ftz fate for different combinations of $t_{\text{on}}$ and $t_{\text{off}}$. For this fraction to achieve 95% requires fairly strict coordination between the timing of modules, with neither event deviating more than about 7 min from $t_{\text{on}} = -20$ min or $t_{\text{off}} = 20$ min (on the order of the mRNA decay rate). Allowing for larger percentages of errors in fate determination significantly weakens constraints on regulatory timing, with a variation of up to 15 min in $t_{\text{off}}$ or $t_{\text{on}}$ still resulting in roughly 50% of cells adopting the appropriate high fate. Right, examples of simulated Ftz traces for a single nucleus with the timing conditions corresponding to the red marks on the heatmap.
Figure S11. Procedure for estimating mRNA and protein expression noise. (A) We extract samples of the noise contribution for an individual sample point \( \sigma_P(P_{\text{total}})dW \) following the method described in the text. We bin the samples by the corresponding \( P_{\text{total}} \) and calculate the mean protein level \( \bar{P}_{k_{\text{total}}} \) within each protein concentration bin \( k \). We divide the samples by \( \sqrt{dt} \) to remove the variance contribution from the \( dW \) term in Eq. S24. We centered the samples from each bin at the corresponding \( \bar{P}_{k_{\text{total}}} \) to generate the pictured histograms. (B) A normal distribution is fitted to each bin in panel (A). (C) Right, we estimate \( \sigma_P(P) \) by fitting a line to the relationship between \( \bar{P}_{\text{total}} \) and variance of the distribution fitted to the corresponding bin in (A) and (B). Left, the equivalent result for mRNA noise, which is calculated identically except that results are binned by \( R_{\text{late}} \) (not pictured).

365 We can perform the above analysis on individual measured traces to produce a large number of sample points of \( \sigma_P(R_{\text{late}})dW \) and \( \sigma_P(P_{\text{total}})dW \). With these data we will aim to estimate \( \sigma_P(R) \) and \( \sigma_P(P) \). We assume the noise characteristics are time invariant, which allows us to pool all samples at all time points and bin them by the corresponding \( R_{\text{late}} \) or \( P_{\text{total}} \). For example, for protein, we treat each protein concentration bin \( k \) as a population of samples of \( \sigma_P(P_{\text{total}})dW \) where \( P_{\text{total}} \) is the mean of the samples in bin \( k \) (Figure S11A). Since we assume \( dW \) is normally distributed with variance \( dt \), we divide all sample values by \( \sqrt{dt} \) and fit a normal distribution to the resulting distribution within each protein concentration bin (Figure S11B). We found that the variances \( \sigma_P(P)^2 \) are quite well approximated by a linear relation \( \sigma_P(P)^2 = aP + b \) (Figure S11C, right). The mRNA variance was estimated similarly and also found to fit a linear relation (Figure S11C, left). Noise was estimated from all available trajectories, regardless of whether they were part of the stripe 4 anterior boundary.

Having estimated the noise, we investigated whether stochasticity could explain the error rate in our predictions of Ftz expression state at gastrulation. We simulated \( N_{\text{sim}} = 100 \) experiments,
Figure S12. Sample traces for nuclei simulated with stochastic dynamics. Top, late mRNA and bottom, total protein traces simulated for individual nuclei obeying the stochastic dynamics in Eq. S24. Gray dashed line in the bottom plot is the threshold to be classified as high Ftz at gastrulation.

Each consisting of $N_{\text{nuc}} = 118$ nuclei evenly split between those deterministically predicted to be on and those deterministically predicted to be off. Specifically, we found the convex hull defined by the experimentally measured initial conditions for nuclei at the stripe 4 anterior boundary, and drew random initial conditions for the stochastically simulated nuclei from a uniform distribution within this hull. We assigned to each simulated experiment $\frac{N_{\text{nuc}}}{2}$ points below the blue surface in Figure 5C and $\frac{N_{\text{nuc}}}{2}$ above the surface without replacement (i.e., every nucleus in every simulated experiment has a unique set of initial conditions). Individual stochastic trajectories were generated using the Euler-Maruyama method, with the modification that protein and mRNA concentrations were forcibly lower bounded at 0 (i.e., random fluctuations that would bring concentrations to negative values were capped to instead bring the concentration to zero). Trajectories were simulated for 20 min until gastrulation and thresholded with the same value as for our deterministic simulations (Figure S12).

From our $N_{\text{sim}} = 100$ simulated experiments, we calculated a distribution of error rates given by

$$\frac{\text{total false positives} + \text{total false negatives}}{\text{total number nuclei}},$$

where we compare the predicted outcomes from deterministic simulations to the “ground truth” of the stochastic simulations.

In Figure S13 we plot the cumulative distribution of error rates for our simulations (black), broken down into false negatives (red) and false positives (green). The dashed vertical lines indicate the experimentally measured error rates with the same color code. Where the vertical lines intersect the corresponding cumulative distributions indicates the probability of measuring an error rate up to that rate. If the system is really described by the stochastic dynamics we have inferred, then the most likely error rates are those that intersect the curves where their slope is highest.

We found that the empirical error rate across 3 embryos was roughly twice that of the most likely error rates from our stochastic simulations corresponding to the middle of calculated cumulative distribution functions (Figure S13, left), with the majority of errors being false negatives. We
Figure S13. Error rates resulting from deterministic predictions made when gene expression is stochastic. Solid lines, cumulative distribution functions for error rates in the prediction of results for $N = 100$ simulated stochastic experiments (using the stochastic differential equation in Eq. S24), when using a deterministic simulation to make the predictions. For each simulation, we predicted the trajectories of $N_{\text{nucl}}$ boundary nuclei evenly partitioned between starting states above and below the surface for classifying high or low Ftz at gastrulation (Figure 5). Dotted vertical lines correspond to empirical error rates for three embryos with $N_{\text{nucl}} = 118$ nuclei (left) across 3 embryos, or excluding one embryo that had a high number of deterministic false negatives (Section 2.3) for $N_{\text{nucl}} = 78$ nuclei (right) across 2 embryos.

knew from observation that one embryo had a large number of false negative predictions (Section 2.3, and, interestingly, if we exclude this embryo from analysis, then the empirical error rate aligns well with what the stochastic model predicts to be most likely (Figure S13, right). This result suggests that many of our prediction errors can likely be attributed to stochastic fluctuations.

Having determined that the error of the model in predicting Ftz expression state at gastrulation is comparable to the error expected when considering gene expression stochasticity, we next turned to the question of whether gene expression stochasticity is expected to play a large role in the long-term Ftz fate of cells in which the early element is no longer active. We ran $N = 100$ simulations beginning from the high steady state and calculated the distribution of first-passage times to particular protein values (Figure S14, left) or to within some Euclidean distance of the opposite steady state (Figure S14, right). These measures give an approximation of the switching rate depending upon how stringently one defines a threshold for switching.

Both trends indicate that switching from high to low occurs at a much faster rate than low to high, with conservative rates of stochastic switching between the high and low Ftz fates of around 2 hr and between low and high fates of approximately 3 hr. For comparison of timescales, Ftz stripes are no longer experimentally detected before the end of germ band extension (Hafen et al., 1984; Carroll, 1985), which occurs approximately 1.5 hours after gastrulation (da Silva and Vincent, 2007). Thus, we find no strong evidence that stochastic switching should contribute significantly to Ftz stripe patterning.
Figure S14. First-passage times suggest that the stochastic switching rate between Ftz fates at steady state occurs on a timescale of hours. Left, time to first passage of a stochastic trace starting from the high (black) or low (red) fate to a given protein value. Right, time to first passage of a stochastic trajectory with a given Euclidean distance from the opposite steady state. In both plots, red denotes simulations beginning in the low Ftz state and black denotes simulations beginning in the high Ftz state.

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