# MicroRNA governs bistable cell differentiation and lineage segregation via a noncanonical feedback

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## ABSTRACT

Positive feedback driven by transcriptional regulation has long been considered a key mechanism underlying cell lineage segregation during embryogenesis. Using the developing spinal cord as a paradigm, we found that canonical, transcription-driven feedback cannot explain robust lineage segregation of motor neuron subtypes marked by two cardinal factors, Hoxa5 and Hoxc8. We propose a feedback mechanism involving elementary microRNA-mRNA reaction circuits that differ from known feedback loop-like structures. Strikingly, we show that a wide range of biologically-plausible post-transcriptional regulatory parameters are sufficient to generate bistable switches, a hallmark of positive feedback. Through mathematical analysis, we explain intuitively the hidden source of this feedback. Using embryonic stem cell differentiation and mouse genetics, we corroborate that microRNA-mRNA circuits govern tissue boundaries and hysteresis upon motor neuron differentiation with respect to transient morphogen signals. Our findings reveal a previously underappreciated feedback mechanism that may have widespread functions in cell fate decisions and tissue patterning.

## INTRODUCTION

Systems-level positive feedback serves as a crucial mechanism for cell cycle progression and cell differentiation by generating switch-like behaviors (Novak *et al*, 2007; Xiong & Ferrell Jr, 2003; Yao *et al*, 2008). These switches are often bistable with respect to external signal, and they give rise to cellular memory or irreversibility of cell fate decisions. For example, during embryonic development, bistability arising from feedback loops in gene regulatory networks generates cellular memory with respect to transient differentiation signals such as morphogens (MacArthur *et al*, 2009; Zernicka-Goetz *et al*, 2009). Whereas several important feedback loops that are responsible for irreversible cell cycle progression involve interactions among proteins (Novak *et al.*, 2007; Xiong & Ferrell Jr, 2003; Yao *et al.*, 2008), the switches in most currently known synthetic and developmental systems are governed by feedback loops mediated by transcriptional activation or inhibition (Balaskas *et al*, 2012; Gardner *et al*, 2000; Höfer *et al*, 2002; MacArthur *et al.*, 2009; Tyson & Novak, 2020; Zernicka-Goetz *et al.*, 2009). Although post-transcriptional interactions, such as those between messenger RNA (mRNA) and microRNA (miRNA), play essential roles in development and other biological processes (Bartel, 2009; Chen & Chen, 2019; de la Mata *et al*, 2015; Esteller, 2011; Ghini *et al*, 2018; Johnston *et al*, 2005; Marcinowski *et al*, 2012; Mendell, 2005), it is unclear if these interactions give rise to feedback loops with the capacity of bistable switches, particularly during mammalian development.

One critical developmental process that requires robust cell fate decisions is boundary formation between adjacent tissues that ultimately perform distinct biological functions. Differentiating cells near the tissue boundary use positive feedback to make robust cell fate decisions in the presence of competing and/or fluctuating positional signals. The feedback mechanisms often involve a pair of mutually inhibiting transcription factors, which form a double-negative feedback loop (one type

of positive feedback loop) (Balaskas *et al.*, 2012; Cotterell & Sharpe, 2010; Edgar *et al*, 1989; Jaeger, 2011; Zagorski *et al*, 2017). However, not all known tissue boundary systems have regulatory networks of this type, and it is unclear whether or how robust fate decisions can be made in systems without such canonical transcriptional feedback mechanisms. One such system is the boundary between two types of motor neurons (MNs) along the rostrocaudal (RC; head-to-tail) axis of the spinal cord in bilateral animals. During embryogenesis, antiparallel gradients of retinoic acid (RA) and fibroblast growth factor members (FGFs) along the RC axis determine the expression patterns of Hoxa5 and Hoxc8 paralogs that, in turn, establish the distinct MN identity and synaptic connectivity of rostral limb-innervating lateral motor column (LMC) neurons and caudal LMC neurons at the brachial segments (Dasen *et al*, 2003; Liu *et al*, 2001). These neurons form a boundary characterized by mutually exclusive expression of Hoxa5 and Hoxc8 transcription factors, respectively (Dasen & Jessell, 2009; Dasen *et al*, 2005). However, the possible existence of a double-negative feedback loop between Hoxa5 and Hoxc8 was challenged by observations that Hoxa5 does not inhibit Hoxc8 in chicken embryos (Dasen *et al.*, 2005; Li *et al*, 2017; Philippidou & Dasen, 2013). Consequently, lack of a known feedback mechanism at the transcriptional level of this system makes it difficult to conceive the mechanism underlying boundary formation between the two MN subtypes. Previous studies have identified two microRNAs, *miR-27* and *miR-196*, that inhibit Hoxa5 and Hoxc8, respectively (Li *et al.*, 2017; Wong *et al*, 2015), but their specific roles in cell fate decisions at the MN-type boundary remain unclear.

Here we used Hox boundary formation in MN subtypes as a paradigm to systematically examine principles of lineage segregation during embryonic development. We found that Hoxa5 and Hoxc8 do not form a feedback loop and their mRNAs exhibit significant cellular overlap, yet they

manifested a mutually exclusive boundary at the protein level. To reveal the feedback mechanism underlying lineage segregation, we constructed a series of mathematical models describing elementary biochemical interactions between mRNA and miRNA, which revealed a broad range of biologically plausible parameters that enable bistability through these mRNA-miRNA interactions. Strikingly, these reaction circuits do not exhibit the loop-like characteristics of most known gene regulatory networks. We derived an intuitive conclusion from mathematical analysis, representing a previously underappreciated feedback mechanism arising from a pair of stoichiometric inhibitors with differential degradation rate constants upon formation of multimeric complexes. This feedback loop between miRNA and mRNA molecules does not require transcriptional control or any other canonical feedback mechanism. We estimate that more than $10^4$ distinct instances of this network topology exist in human cells. Using spatiotemporal modeling, embryonic stem cell differentiation and mouse genetics, we corroborate that: 1) *miR-27* is crucial to maintain stable Hoxa5 expression under transient MN differentiation signaling, and 2) *miR-27* and *miR-196* null embryos exhibit MN boundary disruption. Our study uncovered a family of positive feedback loops with widespread molecular interactions that were not previously known to govern bistable switches, and it reveals an unexpected yet potentially general role for miRNA-mRNA interactions in cell differentiation and development.

## RESULTS

### Lineage segregation of Hoxa5 and Hoxc8 in developing spinal motor neurons

To investigate how Hox proteins interpret and respond to gradients of RA and FGF, we first examined distributional dynamics of Hoxa5 and Hoxc8 within spinal MNs along the RC axis of mouse embryos from embryonic day 9.5 to 12.5 (E9.5~E12.5) (Figure 1 and Figure S1). At E9.5, Hoxa5 was expressed in a subset of cervical segments (prevertebral C4 -> C6), whereas Hoxc8 was absent at this stage. Motor neurons underwent a dynamic boundary formation process from E10.5 to E11.5, with Hoxc8 beginning to be expressed and a small number of Hoxa5$^{on}$/Hoxc8$^{on}$ double-positive MNs being observed at the Hoxa5-Hoxc8 boundary. At E12.5 , Hoxa5$^{on}$/Hoxc8$^{off}$ and Hoxa5$^{off}$/Hoxc8$^{on}$ MNs were sharply segregated into rostro-cervical (C4 -> C7) or caudal-cervical (C8~T1) segments, and virtually no mixed Hoxa5$^{on}$/Hoxc8$^{on}$ hybrid cells were observed (Figure 1B, N≥3 embryos). Thus, Hoxa5 and Hoxc8 manifested dynamic yet sharp and robust segregation during spinal MN development in a timely manner, making it a suitable system for interrogating how a gene regulatory network (GRN) can control lineage decisions at a developing tissue boundary (Figure 1C).

### Segregation of Hoxa5- and Hoxc8-expressing spinal motor neurons is not achieved by mutual inhibition

Lineage segregation of cells at several known tissue boundaries, including the cardinal dorsoventral (DV) progenitors along the neural tube, relies on cross-repressive interactions of transcription factors (TFs). Therefore, one of the most conceivable mechanisms potentially underlying the Hoxa5/Hoxc8 lineage decision at the cervical boundary is a canonical feedback loop formed by Hoxa5 and Hoxc8 mutual inhibition (Figure 2A) (Balaskas *et al.*, 2012; Cotterell

& Sharpe, 2010; Jaeger, 2011; Zagorski *et al.*, 2017). We built a mathematical model based on this regulatory circuit assuming transcriptional cross-repression (T-CR Model, Figure 2A). Unsurprisingly, we found that the model exhibited desirable performance in capturing experimentally-observed boundary formation dynamics, even in the presence of fluctuating RA and FGF signals (Sosnik *et al*, 2016) (Figure 2B~2D, Movie S1). Nevertheless, the T-CR assumption contradicted the observation from chicken embryos that Hoxc8 unilaterally inhibits Hoxa5 (Dasen *et al.*, 2005). To test if Hoxc8 and Hoxa5 exert mutual or unilateral inhibition in a mammalian context, we generated "Tet-ON" inducible Hoxa5::V5 and Hoxc8::V5 tagged mouse embryonic stem cell (ESC) lines (Figure 2E and 2F) (Li *et al.*, 2017). Under conditions of Hoxa5$^{on}$ MN differentiations (Figure 2E), doxycycline treatment on day 4 of differentiation resulted in efficient induction of exogenous Hoxc8::V5 and concomitant suppression of endogenous murine Hoxa5 expression (Figure 2G, quantification in Figure 2H, N=3 from three independent experiments) (see Materials & Methods for details). Similar to the finding from an avian context (Dasen *et al.*, 2005), induction of exogenous Hoxa5::V5 in Hoxc8$^{on}$ differentiated MNs did not repress endogenous Hoxc8 expression (Figure 2I, quantification in Figure 2J, N≥9 embryoid bodies (EBs) from three independent experiments). These results exclude the possibility of a Hoxa5/Hoxc8 cross-repression circuit (T-CR Model).

Next, we built another mathematical model (Transcriptional Unilateral Repression, or T-UR, Model, Figure S2A) to investigate boundary formation under conditions of a unilateral inhibition circuit. We found that slightly noisy morphogen signals gave rise to fluctuating cell lineages and a blurred tissue boundary despite assuming very high cooperativity of gene regulations (Figure S2B and S2C, Movie S2). The poor lineage-decision performance of the T-UR Model relative to our T-CR Model was because the former lacks a positive feedback loop to endow robustness on

cell differentiation. In contrast, the mutual inhibition circuit in the T-CR Model serves as a canonical form of positive feedback loop to generate bistability and enable hysteresis (Figure S2D) (Balaskas *et al.*, 2012). The difference between the two models were robust with respect to the changes of kinetic rates in the models (Figure S2E). Combined, these *in vitro* and *in silico* analyses indicate that the segregation of Hoxa5$^{on}$ and Hoxc8$^{on}$ MNs in the face of known inherently noisy morphogen signals (Sosnik *et al.*, 2016) may be mediated by a mechanism that differs from the canonical design principle involving mutual inhibition of lineage-determining TFs.

**Hoxa5- and Hoxc8-expressing cells delineate a sharp tissue boundary without segregation of their respective mRNAs**

We wondered if an alternative positive feedback loop mechanism (Delás & Briscoe, 2020) involving other TFs upstream of Hoxa5 and Hoxc8 might function as a potential GRN to explain the boundary formation of Hoxa5$^{on}$ and Hoxc8$^{on}$ MNs (Figure S2F). In this case, a clear all-or-none pattern of *Hoxa5* and *Hoxc8* mRNAs near the boundary at E12.5 would be observed. However, unlike the mutually exclusive pattern of Hoxa5 and Hoxc8 protein expression at the boundary, *in situ* hybridization in the same region revealed largely overlapping *Hoxa5* and *Hoxc8* mRNAs within the cervical spinal cord (Figure 3A). To confirm this observation quantitatively at the individual cell level, we performed single-cell RNA sequencing (scRNA-seq) of cervical MNs collected from Hb9::GFP embryos at E12.5 by fluorescence-activated cell sorting (FACS) (Figure 3B). We clustered single-cell transcriptomes using a graph-based approach, which identified major MN subtypes in the cervical spinal cord (known as motor columns) according to known markers (Figure S3) (see Materials & Methods for details) (Chen & Chen, 2019). To focus solely on postmitotic MNs and to simplify our analysis by excluding *Hox* genes poorly or not expressed in

other cell types (i.e. MN progenitors, interneurons, or other cell types), we focused on LMC MNs for further characterization given their strong expression of *Hox* genes (Figure 3C) (Dasen *et al.*, 2005). Similar to the *in situ* hybridization data, we found that *Hoxa5/Hoxc8* mRNAs largely overlapped within individual LMC-MN subtypes, whereas Hoxc8-mediated downstream effector genes *Etv4* (*Pea3*) and *Pou3f1* (*Scip*) were disproportionately distributed in *Hoxc8*$^{+}$ MNs (Figure 3D) as anticipated (Catela *et al*, 2016; Dasen *et al.*, 2005). This result indicates that even though E12.5 cervical MNs have already committed to segregated Hoxa5$^{on}$ or Hoxc8$^{on}$ lineage fate, *Hoxa5/Hoxc8* mRNAs still exhibited extensive cellular co-expression. To analyze this pattern quantitatively along the RC axis of the cervical spinal cord, we used *Hox4~Hox9* mRNA levels as a proxy to estimate the relative MN positions (Figure 3E) (Philippidou & Dasen, 2013). We found that *Hoxa5* and *Hoxc8* were co-expressed in a large subset of cervical MNs over a broad expanse, with a profound increase in the mid-cervical boundary region defined by *Hoxc4~Hoxa9* expression (Figure 3E). These scRNA-seq results confirm our finding of *Hoxa5*/*Hoxc8* mRNA co-expression from *in situ* hybridization. Finally, we performed a lineage tracing experiment using the Hoxc8:Cre;ROSA26-loxp-STOP-loxp-tdTomato line (Carroll & Capecchi, 2015) to reveal any cell in which the Hoxc8 genomic locus was, or had been, active. At E12.5, (Figure 3F) we found that Cre-dependent reporter expression was observed not only in Hoxc8$^{on}$ MNs, but also in a significant subset of Hoxa5$^{on}$ MNs (Figure 3G, N=3 embryos). This lineage tracing experiment further validated that the expression of *Hoxc8* mRNA expression at Hoxa5$^{on}$ MNs in the developing spinal cord, whereas immunostaining revealed no co-expression of Hoxa5/c8 protein (Figure 3G, N=3 embryos). Together, these results demonstrate that Hoxa5 and Hoxc8 proteins are remarkably segregated despite significant overlap of their respective mRNAs. The data imply a key role for post-transcriptional regulation in the lineage decision process, and they argue against

198 the GRNs in the T-CR and T-UR models, both of which assume segregation of *Hoxa5* and *Hoxc8*
199 mRNAs for tissue boundary formation (Figure 3H).

200

201 **Neither a feed-forward loop nor transcription-mediated feedback involving known**
202 **regulatory miRNAs can explain the Hoxa5-Hoxc8 lineage decision at the tissue boundary**

203 Given that broad importance of miRNAs within Hox GRNs across species (Chen & Chen, 2019)
204 and, more specifically, that previous mouse *in vivo* studies have illustrated that correct
205 spatiotemporal expressions of Hoxa5 and Hoxc8 require *miR-27* and *miR-196* respectively (Li *et*
206 *al.*, 2017; Wong *et al.*, 2015), we investigated if incorporating these post-transcriptional regulators
207 into our T-UR Model could improve its lineage segregation performance and resolve its
208 inconsistency with observed mRNA distributions. Upon incorporating all reported miRNA-Hox
209 GRNs into a new model (i.e., the Transcriptional Unilateral Repression with miRNA Regulation,
210 or Tmi-UR, Model, Figure 4A), we found that the inclusion of miRNA-based feed-forward loops
211 facilitated the threshold-like behavior of cell differentiation without requiring complete
212 segregation of *Hoxa5* and *Hoxc8* mRNAs (Figure 4B and C) (Li *et al.*, 2017; Mukherji *et al*, 2011;
213 Osella *et al*, 2011). Nonetheless, the Tmi-UR Model did not generate positive feedback and,
214 consequently, it did not improve lineage-decision or boundary-sharpening processes under
215 conditions of fluctuating morphogen signals (Figure 4C, Movie S3).

216 Previous studies in other systems have reported the possibility of a functional TF-miRNA feedback
217 loop whereby the TF transcriptionally inhibits the miRNA which, in turn, represses the TF (Celià-
218 Terrassa *et al*, 2018; Palm *et al*, 2013; Tsang *et al*, 2007; Yoon *et al*, 2011). Using yet another
219 mathematical model (Transcriptional Unilateral Repression with Transcription and miRNA-

mediated Feedback, or Tmi-FB, Model, Figure 4D), we confirmed that the incorporating two hypothetical transcription-mediated feedback loops between *miR-196* and Hoxc8 and between *miR-27* and Hoxa5 respectively generated a robust lineage decision at the tissue boundary (Figure 4E and F, Movie S4). However, this predicted mechanism requires miRNA expression to be sharply defined. In particular, *miR-196* cannot be expressed in the caudal region in the model (Figure 4E-I), contradicting the observed distribution of *miR-196*, which expands broadly into the caudal region (Kloosterman *et al*, 2006; Wong *et al.*, 2015).

We performed random sampling of parameter values for our four models, and confirmed that these models exhibited either poor performance in lineage segregation or inconsistency with other observations over a wide range of kinetic rate constants (Figure 4G-I). Therefore, the lineage-decision and boundary-sharpening processes of cervical Hoxa5/Hoxc8 MNs are likely governed by a novel post-transcriptional feedback mechanism. In the next section, we describe a new theoretical framework that utilizes the existing gene regulatory network supported by experimental data (Tmi-UR Model) to explain the robust segregation of Hoxa5- and Hoxc8-expressing cells (reflecting performance of the T-CR/Tmi-FB Model).

## Elementary mRNA and miRNA interactions with simple kinetic requirements can generate bistability

To elucidate the possibility that mRNA and miRNA interactions govern feedback-driven bistable switches, we built a series of mathematical models describing elementary biochemical reactions that involve a pair of interacting mRNA and miRNA molecular species (Figure 5). In these models, a binding event occurs when miRNA(s) bind to an mRNA via sequence complementarity, and they

form partially double-stranded complexes (1:$n$ complexes, where $n$ is the number of miRNAs bound to an mRNA). Our models incorporate production of the mRNA and miRNA, as well as their degradation. We assumed that degradation of the complexes involves an elementary step by which either miRNA or mRNA is degraded. This assumption is supported by evidence that both miRNA-mediated mRNA degradation and mRNA-mediated miRNA degradation exhibits multiple turnover (i.e. the miRNA/mRNA can be recycled after the degradation of its binding partner) (Baccarini *et al*, 2011; de la Mata *et al.*, 2015). Note that these models still capture possible scenarios in which both mRNA and miRNA are degraded more rapidly (or more slowly) in the complex.

In the first model (mRNA-miRNA with One Binding Site, or mmi-1, Model), an mRNA molecule has one target binding site for a miRNA molecule (Figure 5A). We found that this model cannot give rise to bistability because it is impossible for the system to have more than one steady state for all positive parameter values. For confirmation, we applied the chemical reaction network theory (CRNT) (Feinberg, 2019) to the model and reached the same conclusion (SI Text). Next, we considered a model in which the mRNA has two miRNA binding sites (mRNA-miRNA with Two Binding Sites, or mmi-2, Model, Figure 5B). Interestingly, we identified a parameter set that supports formation of a bistable switch (Figure 5C, thick curves), an observation consistent with the CRNT and a previous miRNA model (Tian *et al*, 2016). However, the mmi-2 Model did not appear to contain any positive feedback loop, an essential element for most known biological switches. Moreover, the range of kinetic rates enabling bistability and their biological plausibility were unclear.

Next, we used a real algebraic geometry method and numerical bifurcation analyses to derive a set of inequalities that describe the ranges of parameters allowing bistability (a detailed mathematical

analysis is included in SI Text). To examine the intrinsic properties of the regulatory network that enable bistability, we focused on four parameters that describe how fast the mRNA ($a_1$ and $a_2$) and miRNA ($b_1$ and $b_2$) are degraded in the 1:1 and 1:2 mRNA:miRNA complexes, respectively, relative to the degradation rate constants of their single-stranded forms (basal degradation rate constants) (Figure 5B, orange arrows). We found that there is a simple relationship among these rate constants that allows three positive steady states of the system to co-exist (a critical condition for bistability) with certain combinations of the mRNA and miRNA synthesis and basal degradation rate constants (Figure 5C):

$$\frac{a_1}{b_1} < \frac{a_2}{2b_2} \quad (1)$$

This analytical result might serve as a simple and general requirement for bistability arising from mRNA-miRNA circuits, but the stabilities of the three steady states, as well as their biological meaning in terms of molecular concentrations, remained unclear. Therefore, we performed numerical experiments to estimate the parameter range allowing for bistability. With parameters $a_1$, $a_2$, $b_1$ and $b_2$ randomly drawn from a uniform distribution over the interval (0.125, 16) (a range estimated from previous experimental work (de la Mata *et al.*, 2015; Eichhorn *et al*, 2014)), we identified 33.48% parameter sets that generated bistable switches (Figure 5C, thin curves), and there was no constraint on the ranges of the individual parameters in addition to the chosen interval (SI Table S2, Figure S4). This is consistent with the theoretical prediction based on Eq 1. For most of these bistable switches, increasing miRNA concentrations induced a dramatic decrease in the concentration of free mRNA (>3 orders of magnitude), and this change was accompanied by a moderate change, or no change of the total concentration of mRNA (<1 order of magnitude) (Figure 5C and Figure S5). Remarkably, these results suggest that bistable switches may not reflect

prominent changes in total mRNA levels, and that bistability does not require enhanced degradation of both mRNA and miRNA in the complexes. Furthermore, bistability does not seem to require that the degradation rate constants of mRNA and miRNA are in the same range (Figure S5). Nonetheless, Eq 1 and our computational results predict that significantly different mRNA-to-miRNA ratios of degradation rate constants in the 1:1 and 1:2 complexes give rise to bistable switches.

Is this relationship between the two ratios of mRNA and miRNA degradation rate constants (Eq 1, Figure 5C) biologically plausible? We addressed this question by further considering a series of previously reported observations which describe two scenarios of mRNA-miRNA regulations. First, based on multiple turnover of miRNA during target degradation (Baccarini *et al.*, 2011), we assume that miRNA degradation is independent of mRNA degradation and that the miRNA degradation rate remains the same in the complexes ($b_1 = b_2$). Grimson et al. found that target degradation rates arising from two miRNA binding sites are equal to or stronger than the sum of the degradation rates arising from both binding sites individually (Grimson *et al*, 2007) (i.e. $1 < 2a_1 \leq a_2$). In the second scenario, a previous report indicated that the degradation rates of mRNAs containing multiple miRNA binding sites were significantly increased (2-4-fold) with increasing miRNA concentration ($1 < 2a_1 \leq a_2$), whereas this increase was not observed for mRNAs containing single binding sites for the same miRNAs (de la Mata *et al.*, 2015). Consequently, miRNA-mediated target degradation is cooperative when multiple miRNA molecules bind to the same mRNA molecule, consistent with other studies (Grimson *et al.*, 2007). Interestingly, in this scenario, the miRNA degradation rates were reduced with increasing concentration of miRNA ($b_1 > b_2$) (de la Mata *et al.*, 2015; Ghini *et al.*, 2018). Thus, the effectiveness of target-induced miRNA degradation in mammalian cells is sensitive to the mRNA:miRNA concentration ratio.

Therefore, the reverse ratios between the mRNA and miRNA degradation rate constants in different forms of complexes appear to be biologically plausible. Accordingly, based on these previous studies, the simple kinetic relationship we describe in Eq 1 reflects a wide range of realistic parameters. Note that this relationship encompasses other plausible kinetics that allow bistability (e.g. $1 > b_1 > b_2$, or reduced miRNA degradation through multimeric complex formation).

Finally, we built a model for an mRNA with three miRNA binding sites (mRNA-miRNA with Three Binding Sites Model, or mmi-3, Model, Figure 5D). Through computational analysis with parameter values randomly drawn from a uniform distribution, we found that the fraction of kinetic rate constants that gave rise to bistable switches (50.81%) was even greater than for the mmi-2 Model (Figures 5D and S6). This result indicated that the increased complexity of the reaction network further reduces the requirement for specific rate constants to generate bistability.

Together, the mmi-2 and mmi-3 Models demonstrate that a simple and biologically plausible relationship among kinetic rates involved in mRNA-miRNA interactions enables formation of bistable switches. However, the interaction networks (Figures 5B and D) do not appear to contain any positive feedback loop, i.e. the network motif considered essential for biological switches and boundary formation (Goldbeter, 2018; Novak *et al.*, 2007; Xiong & Ferrell Jr, 2003). This observation prompted us to investigate how to interpret this phenomenon in a context that encompasses a feedback loop, and how such a theoretical framework could be used to better understand specific biological systems such as the differentiation of MNs.

## An intuitive interpretation of our mathematical analyses reveals a large emerging family of noncanonical feedback loops

Since the condition for bistability involves relationships among degradation rate constants, one possible explanation incorporating a feedback mechanism is that mRNA and miRNA are mutually inhibitive by triggering degradation of each other, thereby forming a double-negative feedback loop. However, this explanation does not reflect the requirement of two forms of complexes, nor does it accurately describe the kinetic relationship (Figure 5C, Eq 1) which suggests that either or both RNA molecules can be 'protected' by complex formation, reducing degradation rates without loss of bistability (Figure 5C and D). We sought to find a unifying and accurate way to resolve this discrepancy by converting the chemical reaction networks into simple influence diagrams, which are commonly used to describe gene regulatory networks using intuitive interpretation.

Since stoichiometric inhibitors are ubiquitous in biology (Ferrell & Ha, 2014; Hopkins *et al*, 2017), it is useful to view the two RNA molecules as a pair of stoichiometric inhibitors (sequesters or sponges) that can mutually reduce their free-form concentrations. Here, the single-stranded (free) forms are considered the 'active' forms. For example, in the mmi-1 Model, if the degradation rate constants of mRNA and miRNA are balanced in the complex (with respect to the degradation rates of their single-stranded forms) ($a_1/b_1 = 1$), then they simply sequester each other (Figure 6A, left). These canonical stoichiometric inhibitions are not feedback loops and they do not generate bistability, as noted in a previous study (Hopkins *et al.*, 2017). Furthermore, even if complex formation enhances or reduces degradation of both molecules with the same magnitude ($a_1/b_1$ remains as 1), they are still influenced symmetrically by a single regulatory mechanism. Finally, if the two molecules in the complex exhibit different degradation rate constants relative to their respective free forms ($a_1/b_1 \neq 1$), then the symmetry of the inhibition is broken. Accordingly, the

molecule with the lower relative degradation rate constant is a more efficient inhibitor because it is recycled more frequently during degradation events (Figure 6A, right). Consequently, inhibition becomes directional and, for each case of directional inhibition, complex formation favors either mRNA or miRNA. Therefore, although the regulatory asymmetry is unidirectional, a feedback loop is still absent from the system.

If mRNA and miRNA form two types of complexes (1:1 and 1:2), i.e., the mmi-2 Model, the molecular influence of these two types of RNA on each other (underpinned by the reaction network) become remarkably diverse and they depend upon the relationships among four relative degradation rate constants (Figure 6B). If inhibition of the mRNA and miRNA is symmetrical ($a_1/b_1 = a_2/b_2 = 1$), the influence of the mRNA and miRNA on each other is nondirectional, albeit governed by the formation of the two complexes. If this symmetry is broken and the unbalanced degradation rate constants of the two complexes favor the same RNA species (Figure 6B cyan arrow, and C), then the influence is unidirectional. However, if the symmetry of the two inhibitory processes is broken in opposite directions and the two complexes favor different RNA species, respectively (Figure 6B magenta arrow and D), then a double-negative feedback loop emerges (Figure 6D). Notably, a defined threshold ($2a_1/b_1 = a_2/b_2$) marks the qualitative transition from symmetrical stoichiometric inhibitions (sequestration) to the feedback loops that enable bistability.

This intuitive explanation informed by influence diagrams captures the essence of the key kinetic relationship that only constrains the ratios of the degradation rate constants between mRNA and miRNA, rather than their absolute values. We found that this explanation of stoichiometric inhibition with broken symmetry also applies to our mmi-3 Model, in which each mRNA molecule has three miRNA binding sites, although we were unable to describe the threshold between

stoichiometric inhibition and the double-negative feedback loop by means of a simple analytical form. Thus, the formation of bistable switches requires deviation from symmetrical inhibition (Figure 6E, right, orange dot) and the absence of unidirectional asymmetry of the rate constants (Figure 6E, left and right, cyan arrows), and both of these requirements are consistent between the mmi-2 and mmi-3 Models.

Since the mRNA-miRNA interactions in the mmi-2 and mmi-3 Models only represent elementary biochemical reactions, we expect that this network topology is widely deployed in gene regulation. By analyzing predicted miRNA-binding sites in human mRNAs (see Methods), we found that our mmi-2 Model describes 9,571 mRNA-miRNA pairs and up to 122,885 regulatory networks with distinct binding sites, whereas our mmi-3 Model describes 1,250 mRNA-miRNA pairs and up to 93,049 networks (Table 1). Strikingly, genes that contain two or three targeting sites from the same miRNA mostly belong to cell signaling pathways that are involved either in development or action potential firing processes, which are well known for their irreversible and "all-or-none" characteristics of bistability (Figure S6C and S6D). Given the biological relevance of the kinetic relationship (de la Mata *et al.*, 2015; Ghini *et al.*, 2018; Grimson *et al.*, 2007), this analysis implies that the noncanonical feedback mechanism we have derived in this study is applicable to a wide range of gene regulatory networks at the post-transcriptional level.

**miRNA confers hysteresis in the response of Hoxa5 to RA signaling**

Next, we assessed if gene regulation in the MN differentiation system involves networks described by our mmi-2 or mmi-3 models. To do this, we searched for conserved miRNA target sites of Hoxa5 and Hoxc8 using TargetScan (Agarwal *et al*, 2015). We identified three conserved

predicted targeting sites for *miR-27* in the *Hoxa5* 3' untranslated region (UTR), whereas *miR-196* is predicted to have four conserved targeting sites in the *Hoxc8* 3' UTR (Figure S7A). This indicates that the mRNA-miRNA interactions in this MN differentiation system satisfy the theoretical requirement (in terms of the network topology) for a bistable switch.

Next, we tested if the process of MN differentiation exhibits characteristics of a bistable switch. To establish a reliable *in cellulo* model for assaying how Hoxa5 interprets RA signal during ESC differentiation with or without the miRNA/Hox GRN (Figure 7A and S7B), we treated ESCs with a gradient dosage of RA concentration from high (1 μM) to low (100 nM), which reliably resulted in a correspondingly proportional high to low number of Hoxa5$^{on}$ cells (Figure S7C and quantification in Figure S7D). Exposure to [RA]$^{high}$ for 72 hours during differentiation induced robust Hoxa5 expression when assayed at 96 hours, representing a stage when Hoxa5$^{on}$ MNs become postmitotic. Interestingly, [RA]$^{high}$ exposure for only 48 hours at the progenitor stage also elicited persistent Hoxa5 expression. Moreover, expression of Hoxa5 was not compromised by switching treatment from [RA]$^{high}$ to [RA]$^{low}$ after 48 hours, nor by blocking RA signaling at 48 hours by addition of the pan RA inhibitor (RARi: AGN 193109, Tocris), and this cellular memory was recapitulated in the [RA]$^{low}$ condition (Figure 7B and C, quantification in Figure 7D, N≥3 EBs from three independent experiments). This experiment demonstrated that expression of Hoxa5 during ESC differentiation manifests hysteresis to transient RA signal, which mimics fluctuating morphogen signal during embryonic development (Sosnik *et al.*, 2016).

Subsequently, we examined if *miR-27* is necessary to establish the bistable switch of the RA-induced Hoxa5 response. As *miR-27a/b*$^{-/-}$ ESCs exhibited poor MN differentiation efficiency (Li *et al.*, 2017), we used *iMir27* sponge ESCs (*iMir27SP*) instead to decoy *miR-27* after the progenitor stage (Figure 7E). Both control *iScrmSP* and *iMir27* sponge cells exhibited similar

proportions of Hoxa5$^{on}$ cells upon [RA]$^{high}$ treatment (Figure 7F). However, in conditions where [RA]$^{high}$ was switched to [RA]$^{low}$ after 48 hours (Day0~2), the presence of*iMir27* sponge induced a significant decrease in Hoxa5$^{on}$ cells, even though MN fates were not compromised based on Isl1(2) immunostaining (Figure 7F and quantification in 7G, N≥3 EBs from three independent experiments). This finding indicates that MN differentiation hysteresis in the presence of transient RA signal was abolished upon loss of *miR-27*. Not only are these experimental observations consistent with our general theoretical analysis of miRNA-mRNA circuits (mmi-3 Model), but they were also captured by a specific set of model parameters describing the RA-induced differentiation of ESCs under both unperturbed and perturbed conditions (Figure 7H). Therefore, our theoretical framework offers a potential explanation for the robustness of cervical spinal cord patterning. Together, these experiments provide consistent support for our hypothesis that *Hox* expression is resistant to morphogen fluctuation and that the noncanonical mRNA-miRNA feedback we report here might play an essential role in facilitating cell fate decisions of MNs.

**Noncanonical mRNA-miRNA feedback explains mechanisms underlying motor neuron differentiation and pattern formation in the spinal cord**

We wondered if our theoretical framework of mRNA-miRNA circuitry could explain MN differentiation patterns in the developing spinal cord. To test the possibility that *miR-27* and *miR-196* regulate cell fate decisions at the tissue boundary through positive feedback, we built a spatiotemporal model describing the developing MNs near the boundary of the Hoxa5 and Hoxc8 expression domains (mmi-S Model, Figure 8A). In addition to the antiparallel RA and FGF gradients, the mmi-S Model incorporates known *Hox* GRNs, as well as the interactions of miRNAs and mRNA through multiple binding sites, which have been verified experimentally (Figure S7A)

(Li *et al.*, 2017; Li *et al*, 2010). We assumed that for each pair of miRNA and mRNA, the two RNA molecules serve as stoichiometric inhibitors of each other in an asymmetrical and bidirectional fashion, as described above and depicted in Figure 6D and Figure S8A. Our mmi-S Model recapitulated the observations that robust cell fate decisions are reflected in a mutually exclusive expression pattern of Hoxa5 and Hoxc8 at the protein level, but not at the mRNA level (Figure 8B). Interestingly, boundary formation was achieved despite the presence of transient and noisy morphogen signals (Figures 2B, 8B, Movie S5). The mmi-S Model revealed that this particular MN differentiation system exhibited three types of steady state: Hoxa5$^{on}$Hoxc8$^{off}$ (rostral LMC neuron fate), Hoxa5$^{off}$Hoxc8$^{on}$ (caudal LMC neuron fate), and Hoxa5$^{off}$Hoxc8$^{off}$ (undifferentiated) (Figure 8C). Notably, these three steady states could coexist when concentrations of both RA and FGF were low, thus forming a tristable system (Figure 8C). This property not only enabled robust fate decisions in the presence of competing morphogen signals, but also provided additional robustness against receding signals. Transient morphogen signals in the developing spinal cord have been observed in both dorsoventral and rostrocaudal patterning due to the pronounced cell proliferation rate and axis elongation during development (Balaskas *et al.*, 2012; Ensini *et al*, 1998). In this scenario, the GRN described by the mmi-S Model renders progenitors insensitive to signaling fluctuations and confers hysteresis. Overall, the performance of this model in terms of lineage segregation and consistency with experimental data is better than all other models considered in this study (Figure S8B-C).

To examine the roles of mRNA-miRNA interactions in MN patterning during development, we simulated the mmi-S Model in the absence of *miR-27* or *miR-196*. The model predicted that loss of *miR-27* compromised fate decisions and tissue patterning, with Hoxa5 and Hoxc8 presenting overlapped expression at both mRNA and protein levels in the caudal domain (Figure 8D, Movie

S6). This outcome could be attributed to loss of the Hoxa5$^{off}$Hoxc8$^{on}$ steady state, which was replaced by the double-positive Hoxc5$^{on}$Hoxc8$^{on}$ state even under the condition of low morphogen concentrations (Figure 8E). Moreover, our mmi-S Model predicted that loss of *miR-196* shifts the Hoxa5-Hoxc8 boundary in the rostral direction (Figure 8F and 8G, Movie S7).

Next, we compared our mmi-S model with an alternative model in which each mRNA and miRNA pair do not form a feedback loop according to our theoretical framework (altered mmi-S Model, Figure S8D). Due to noisy RA and FGF signals, cellular identities near the tissue boundary manifested significant temporal fluctuations and were not segregated at the steady state, reflecting the loss of robust cell fate decisions (Figure S8E). Together, these results indicate that feedback between mRNA and miRNA is essential for the robustness of cell fate decisions near the boundary, as well as for the stability of boundary positioning.

**_miR-27_ and _miR-196_ govern the sharp and robust Hoxa5-Hoxc8 boundary in the spinal cord**

To address if the predicted function of the miRNA-mRNA circuitry as a bistable switch for Hox proteins explains boundary formation in MNs, we conducted gain-of-function and loss-of-function experiments. First, we developed two 'Tet-ON' inducible ESC lines (*imiR-27b* and *imiR-196a*) in which the primary miR-27b or miR-196a sequences were inserted into the 3' UTR of an inducible GFP construct (Figure 9A and 9B). Induced expression of *miR-27a* in Hoxa5$^{on}$ MNs resulted in efficient suppression of Hoxa5 expression (Figure 9C, quantification in Figure 9D, N≥3 EBs from three independent experiments). Similarly, overexpression of *miR-196a* in Hoxc8$^{on}$ MNs led to a robust reduction of Hoxc8$^{on}$ cells (Figure 9E, quantification in Figure 9F, N≥3 EBs from three independent experiments). In both cases, the generic fate of MNs revealed by Hb9 or Isl1(2)

immunostaining was not affected (Figure 9C and E, quantifications in Figure 9D and F, N≥3 EBs from three independent experiments), indicative of specific miRNA-mRNA feedback loops operating separately within the miR-27/Hoxa5 and miR-196/Hoxc8 GRNs.

Next, we investigated if *miR-27* and *miR-196* are required for the sharp Hoxa5-Hoxc8 boundary in the spinal cord (Figure 10A and 10B). For *miR-27*, we utilized miR-23–27–24 cluster double-knockout (DKO) mouse embryos (Li *et al.*, 2017) to scrutinize the Hoxa5-Hoxc8 boundary segregation process in the spinal cord. A significant number of MNs near the Hoxa5-Hoxc8 boundary in these DKO mouse embryos manifested mixed expression of Hoxa5 and Hoxc8, a cellular phenotype rarely observed in the control embryos at the same stage of development (Figure 10C). Furthermore, we observed intermingled Hoxa5$^{on}$Hoxc8$^{off}$ and Hoxa5$^{off}$Hoxc8$^{on}$ cells in regions where the control embryos showed clear segregation of these two lineages (Figure 10C and quantification in Figure 10D. N=3 embryos), suggesting that the robustness of the cell fate decision is correlated with the robustness of tissue patterning, and that miRNAs such as *miR-27* significantly contribute to these phenotypes.

To interrogate the role of *miR-196* in maintaining the precise boundary between Hoxa5 and Hoxc8, we examined Hoxa5 and Hoxc8 protein expression upon loss of all three miR-196 paralogs (196a1, 196a2, and 196b), as they act redundantly to pattern the mid-thoracic region (Figure 10B) (Wong *et al.*, 2015). Compared to controls, Hoxc8 protein was expanded profoundly in the rostral Hoxa5$^{on}$ MNs of miR-196 double (miR-196a2/b) and triple (miR-196a1/a2/b) KO embryos at E12.5 (Figure 10E and quantification in Figure 10F, N=3 embryos). Notably, this phenotype is consistent with a report that loss of *miR-196* leads to collective upregulation of numerous *Hox* target genes in the trunk region of mice, which induced impairments in vertebral number and vertebral identity (Wong *et al.*, 2015). Furthermore, all of our *in vivo* observations of control embryos or those lacking *miR-*

513 *27* or *miR-196* are consistent with our predictions from the mmi-S Model (Figure 8B-F). Taken
514 together, these findings indicate that miR-27/Hoxa5 and miR-196/Hoxc8 feedback loops are
515 required to maintain a sharp and precise postmitotic MN boundary in the spinal cord (Figure 10G).

516

517 **DISCUSSION**

518 **Role of miRNAs in robust cellular responses**

519 One of the most fascinating questions in biology is how cell fate determination is generally robust
520 in view of fluctuating environmental challenges during embryonic development. Waddington
521 termed this process "canalization", with greater net canalization during development resulting in
522 less phenotypic variation among individuals in a population (Waddington, 1942). Numerous
523 studies have shown that miRNA confers robustness on gene expression through canalization
524 mechanisms, such as feed-forward loops (Ebert & Sharp, 2012; Li *et al*, 2009; Osella *et al.*, 2011;
525 Siciliano *et al*, 2013). miRNA can also generate thresholds for gene expression that enhance
526 nonlinearity of the dose response (Mukherji *et al.*, 2011). In this study, we found that mRNA-
527 miRNA interactions can generate bistable switches, representing a form of cellular memory,
528 through a feedback loop mechanism that was not previously identified. The simple molecular
529 composition and biologically plausible kinetics of these interactions imply that this feedback loop
530 mechanism could act broadly as a GRN motif to generate cellular memory. Previous studies have
531 shown that combined with TF-mediated miRNA control, miRNA-mediated translational inhibition
532 can be involved in feedback loops (Lu *et al*, 2013; Tsang *et al.*, 2007). Our work reveals a distinct
533 feedback mechanism at the post-transcriptional level, which endows robustness on cellular
534 phenotypes in concert with the noise attenuation mechanisms governed by feed-forward loops (Li
535 *et al.*, 2017; Osella *et al.*, 2011).

## What triggers mRNA-miRNA-mediated bistable switches?

Through our proposed mRNA-miRNA feedback mechanism, we show that altered synthesis or basal degradation rates of mRNA or miRNA can trigger a switch in the concentrations of free mRNA and free miRNA molecules. In the system of MN development we consider here, this switch is triggered by upregulation of *Hox* genes through transcriptional activation induced by morphogen signals. However, a myriad of sources could trigger the switch between free mRNA and miRNA concentrations in such circuits. For example, multiple types of mRNA may compete for a single pool of miRNA (the competitive endogenous RNA hypothesis) (Tay *et al*, 2014). In this case, a transcriptional change in a competing mRNA can trigger the switch by altering the balance of mRNA and miRNA in the feedback circuit. The switch can also be triggered by changing mRNA or miRNA stability via mechanisms external to the circuit.

## Cooperativity of miRNA mediated regulations

We have shown that a key component of the mRNA-miRNA feedback mechanism is formation of multiple types of mRNA-miRNA complexes. In addition, this feedback mechanism requires that the complexes have distinct ratios of mRNA and miRNA degradation rates. This kinetic property can be achieved through cooperativity in miRNA-mediated mRNA degradation (de la Mata *et al.*, 2015). It also indicates that distinct miRNA molecules can form a feedback circuit with an mRNA if they synergistically reduce the stability of that mRNA. Under this condition, the feedback mechanism does not require multiple binding sites of one type of miRNA on an mRNA, so our

theoretical framework can be applied to much broader regulatory circuits involving mRNA-miRNA interactions.

Our proposed kinetic requirement for bistability encompasses, but does not depend on, target-directed miRNA degradation (TDMD), whereby specialized target RNAs selectively bind to miRNAs and induce their decay. Although the detailed mechanism underlying TDMD is not yet entirely clear, structural and biochemical insights indicate that 3'-end miRNA/mRNA complementary sequence-matching is a key determinant regulating miRNA activity via 3'-remodeling and/or degradation (Sheu-Gruttadauria *et al*, 2019). In this scenario, *miR-196* and Hoxc8 not only have multiple targeting sites, but also display extended matching 3'-end sequences (Figure S7A), which might reinforce the reciprocal inhibition between *Hoxc8* and *miR-196*. This topic warrants future verification of potential TDMD of the *Hoxc8*/*miR-196* pair. Experimentally, results from us and others clearly show that: 1) Hoxa5 and Hoxc8 have multiple predicted targeting sites for *miR-27* and *miR-196*, respectively (Li *et al.*, 2017; Li *et al.*, 2010; Yekta *et al*, 2004); and 2) luciferase assay reveals that expression levels of Hoxa5 and Hoxc8 are only significantly reduced upon mutation of multiple sites (Li *et al.*, 2017; Yekta *et al.*, 2004). These results support our modeling that the bistable switch from mRNA-miRNA feedback likely operates via formation of multiple types of mRNA-miRNA complexes. Although we have not established exactly how the dynamic bistable switch of miR-27/Hoxa5 and miR-196/Hoxc8 is balanced at the molecular level, we have clearly shown that this miRNA-mRNA bistable switch constitutes a cell memory that is robust in light of fluctuating environmental signals. It is tantalizing to explore how broadly this miRNA-mRNA bistable switch is exerted in other cell fate decisions during embryonic development.

## Multifaceted functions of stoichiometric inhibition

It was shown previously that whereas the stoichiometric inhibition alone is insufficient to serve as a feedback loop, but it often contributes to feedback loop formation (Hopkins *et al.*, 2017). Interestingly, simple stoichiometric inhibition can also work as a switch in stochastic systems (Lord *et al*, 2019). Our results show that when kinetics (e.g. the ratio of degradation rates) deviate from a balanced condition in terms of two mutual inhibitors, feedback-like reaction networks can emerge and they can generate bistable switches deterministically. These kinetic variations not only create bistable systems via bifurcation, but they also determine the existence of feedback in a quantitative and continuous fashion. Bistability arising from biochemical reaction networks without explicit feedback loops have been observed based on mathematical models of protein kinase cascades (Markevich *et al*, 2004). Together, these results highlight the importance of kinetics and the structures of chemical reaction networks when analyzing GRNs, and they reveal an important limitation of using a set of directed edges to describe GRNs.

## mRNA-miRNA feedback and the diverse molecular functions of miRNA

The canonical molecular functions of miRNA include mRNA destabilization and translational inhibition (Bazzini *et al*, 2012; Djuranovic *et al*, 2012; Petersen *et al*, 2006). In our models of mRNA-miRNA feedback, a wide range of parameter values enable bistability, such that this feedback mechanism can use both or either of those molecular functions to achieve bistability. This implies that the switch-like property may be a functional trait that has been selected through diverse molecular mechanisms, and it may serve as a unifying performance objective for a broad range of mRNA-miRNA systems, regardless of their molecular functions. Nonetheless, our

analysis shows that the feedback mechanism requires strong mRNA-miRNA binding (small $K$) and regulation of the degradation rates in the complexes, indicating that the canonical functions of miRNA (translational inhibition and mRNA degradation) and TDMD both contribute to how the bistable switch is driven synergistically by the feedback mechanism. Moreover, the relative half-lives for individual miRNAs can vary among cell types (Kingston & Bartel, 2019), implying that the turnover dynamics of mRNA-miRNA during MN differentiation should be considered in our predictive model, which might contribute to balancing the switch between miRNA and target mRNA. Experiments in which the dynamics of miRNA metabolism during spinal MN development are assessed using steady-state metabolic labeling of *mir-27* and *mir-196* would shed light on this topic.

**Tissue-level boundary sharpening**

While the performance of our model is similar to that of the known GRNs responsible for robust boundary formation (Balaskas *et al.*, 2012; Cotterell & Sharpe, 2010; Goldbeter *et al*, 2007; Zagorski *et al.*, 2017), it reveals a previously underappreciated boundary formation mechanism at the post-transcriptional level. Models based on GRNs with feedback loops focus on interpretation of positional signals with autonomous mechanisms for single cells, but sharp boundary formation at the tissue level often involves interactions among cells, including cell migration and induced lineage switching (Addison *et al*, 2018; Cooke *et al*, 2001; Dahmann *et al*, 2011). Nonetheless, unambiguous cell fate decisions may serve as a foundation for further fine-tuning of tissue boundaries through intercellular interactions. In addition to the feedback mechanism proposed in this study, adopting mRNA-miRNA circuits may allow progenitors to maintain plasticity at the early stage for a diverse choice of subsequent cell fates (Chakraborty *et al*, 2020), and applying

the miRNA-mRNA bistable system is advantageous for post-mitotic cells to ensure robustness against fluctuating environments during tissue morphogenesis. We argue that miRNA represents the best arbiter to coherently reconcile these requirements for plasticity and robustness during development. We envisage that future experiments to scrutinize the dynamics of miRNAs and their target interactions at detailed temporal and spatial resolutions by means of single-cell technology will reshape our concept of how cells adopt plastic but robust fates.

## MATERIALS AND METHODS

### Mouse ESC culture and MN differentiation

*Hb9::GFP, iHoxc8-V5, iHoxc8-V5, imiR-ScrmSP, imiR-27SP, imiR-196a OE,* and *imiR-27b OE* ESCs were cultured and differentiated into spinal MNs as previously described (Yen *et al*, 2020). In some cases, caudal LMC neuron differentiation was achieved by including 100 ng $ml^{-1}$ bFGF together with reduced concentrations of RA and SAG at Day2 of differentiation (Tung et al., 2019). All cell lines used in this study are subjected to regular mycoplasma tests.

### Immunocytochemistry

Commercially-available primary antibodies and antibodies gifted by J Dasen, H Wichterle, and TM Jessell are described in the table of reagents.

### miRNA *in situ* hybridization

Sections were fixed in 4% paraformaldehyde and acetylated in acetic anhydride/triethanolamine, followed by washes in PBS. Proteinase K treatment was skipped for post-immunostaining. Sections were pre-hybridized in hybridization solution (50% formamide, 5 SSC, 0.5 mg $ml^{-1}$ yeast

tRNA, 1 X Denhardt's solution) at room temperature, before being hybridized with 3'-DIG or FITC-labeled LNA probes (3 pmol) (LNA miRCURY probe; Exiqon) at 25 ºC below the predicted Tm value. After post-hybridization washes in 0.2 SSC at 55 ºC, the *in situ* hybridization signals were detected using the NBT/BCIP (Roche) or Tyramide Signal Amplification system (Perkin-Elmer) according to the manufacturer's instructions. Slides were mounted in Aqua-Poly/Mount (Polysciences Inc.) and analyzed by using a Zeiss LSM710 Meta confocal microscope.

**Mouse crosses and *in vivo* studies**

Wild type (WT) C57BL6/J male mice were mated with WT female mice to generate embryos to assess Hox proteins. *miR-23~27~24* DKO mice were generated by crossing *miR-23a~27a~24-2*$^{+/-}$*;miR-23b~27b~24-1*$^{+/-}$ male mice with *miR-23a~27a~24-2*$^{+/-}$*;miR-23b~27b~24-1*$^{+/-}$ female mice for experimental analysis. Similarly, the *miR-196* DKO and *miR-196* TKO lines were generated by crossing *miR-196a1*$^{+/-}$*;miR-196a2*$^{-/-}$*;miR-196b*$^{-/-}$ male mice with *miR-196a1*$^{+/-}$*;miR-196a2*$^{-/-}$*;miR-196b*$^{-/-}$ female mice. Control mice were generated by crossing WT male mice with WT female mice to generate the relative staged embryos for comparison. Mice were mated at the age of 8~12 weeks and the embryo stage was estimated as E0.5 when a copulation plug was observed. Embryos were analyzed between E9.5~E13.5. All of the live animals were kept in an SPF animal facility, approved and overseen by IACUC Academia Sinica.

**Lineage tracing and *in vivo* studies**

Hoxc8 lineage tracing experiments were performed by crossing Hoxc8-IRES-Cre mice (a kind gift from M Capecchi's lab) with Ai14 (ROSA26-loxp-STOP-loxp-tdTomato) mice (from JAX stock #007908) to generate Hoxc8:Cre;ROSA26-loxp-STOP-loxp-tdTomato (lsl-tdTomato) embryos for

experimental analysis. Mice were mated at the age of 8~12 weeks and the embryo stage was estimated as E0.5 when a copulation plug was observed. Embryos were analyzed between at E12.5. All of the live animals were kept in an SPF animal facility, approved and overseen by IACUC Academia Sinica.

**Single-cell sample preparation and RNAseq**

Hb9::GFP (Mnx1::GFP) (JAX stock #005029) transgenic male mice were mated with B6 mice to produce embryos in which all MNs were labeled with green fluorescence. Mating was confirmed by the presence of a vaginal plug the next morning and defined as embryo stage E0.5. For sample collection, E12.5 mouse embryos were euthanized with $CO_2$ and dissected in Leibovitz's L-15 medium (Gibco, 11415064) to isolate brachial spinal cords (segment C2-T1). Tissue dissociation was carried out by means of enzymatic and mechanical approaches using a Neural Tissue Dissociation Kit (P) (Miltenyi Biotec,130-092-628) and a gentleMACS dissociator (Miltenyi Biotec,130-093-235) according to the manufacturers' instructions. Dissociated cells were resuspended in N2B27/DMEM-F12 and neurobasal medium containing N2 (Life Technologies, 17502048) and B27 (Life Technologies, 17504044), 1% Penicillin-streptomycin, 2 mM L-glutamine, 0.2 M β-mercaptoethanol and 0.5 μM ascorbic acid, supplemented with 1% inactivated fetal bovine serum (FBS) and filtered through a 70-μm strainer (Falcon, 352350). Dissociated cell suspension was subjected to sorting using a BDFACSAria III cell sorter (BD BioSciences, USA) with a 85 μm diameter nozzle and 45 sheath pressure. $GFP^+$ cells were collected into DMEM plus 1% FBS and immediately processed for cell counting and single-cell isolation. The quality of cells was assayed by measuring live versus dead cells (Thermo Fisher Scientific, L3224) and checked

for aggregation using a Countess II Automated Cell Counter (Thermo Fisher Scientific, AMQAX1000).

Single-cell suspension was loaded onto a 10x Genomics Single Cell 3' Chip (10x Genomics, Pleasanton, CA) and subjected to single-cell isolation, cDNA synthesis and library construction following the Chromium Single Cell 3' v3.1 protocol (10x Genomics, PN-1000121). The single-cell library was sequenced using a NextSeq 500/550 platform with pair-ended reads (PE150: Read 1: 28 bp; Read 2: 122 bp).

**Data pre-processing and clustering of cell types**

The raw single-cell RNAseq dataset was processed using the Cell Ranger pipeline (version 3.1.0, 10x Genomics) with standard procedures for demultiplexing, mapping to the mm10 reference via STAR aligner, filtering, barcoding and UMI counting. The generated expression matrix (cell x gene) was imported into the R platform for downstream analyses.

We performed quality filtering, normalization and scaling, dimensionality reduction and cell clustering using Seurat package version 2.3.4. Cells fitting the following criteria were retained for further analyses: 1300-8000 genes and <72200 unique molecular identifiers (UMI), with the upper limits set at the $97^{th}$ percentile, beyond which cells were regarded as outliers. Cells with more than 10% mitochondrial-associated UMI were considered damaged and were removed from the dataset. The dataset was log-normalized and multiplied with a scaling factor of 10,000. Highly variable genes across cells were selected by means of a binned mean-variable plot and subjected to principal component (PC) analysis. To identify significant PCs for clustering analysis, we used both an

716 elbow plot and a jackstraw test (Chung & Storey, 2015). For the former, the elbow lay between the
717 $30^{th}$ and $40^{th}$ PCs, indicating PCs after the first 40 PCs contributed little to overall variation, so we
718 used the first 40 PCs for further analysis. The jackstraw test demonstrated that all 40 initial PCs
719 were significant with a threshold of 0.001. Cells were clustered using the default Louvain
720 algorithm in the FindClusters function with a resolution of 0.18, and visualized on UMAP. Motor
721 neurons (MNs) and non-motor neurons were initially distinguished based on expression of generic
722 MN markers such as *Mnx1* and the cholinergic genes *Chat*, *Slc18a3*, and *Slc5a7*. Only clusters
723 expressing MN markers were considered and further analyzed using the same pre-processing
724 procedures as described above, but with a cluster resolution set to 0.28. Motor columns were
725 specifically defined according to their enrichment of known motor column markers. The LMC
726 motor column expressed *Foxp1* and *Aldh1a2* and was further subdivided into $Isl1^{+}$ (LMCm) and
727 $Lhx1^{+}$ (LMCl) groups, whereas the MMC motor column expressed *Lhx3*, *Lhx4* and *Isl1*.
728
729 **LMC subclustering**
730 Clusters expressing known LMC markers were defined and clustered as described above, except
731 that the first 30 PCs were used and cluster resolution was set to 0.78. Violin plots were generated
732 to visualize log-normalized UMI counts for motor pool markers such as *Etv4* and *Pou3f1* in
733 individual cells.
734
735 **Quantification of the $Hoxa5^{on}$/$Hoxc8^{on}$ cell ratio**
736 Our analysis focused on LMC motor neurons. We used the SubsetData function in Seurat to retain
737 annotated LMC clusters for quantification. We binarized expression of *Hoxc4*, *Hoxa5*, *Hoxa7*,
738 *Hoxc8* and *Hoxc9* based on the frequency distributions of these genes in the dataset. Gene

739 expression was assumed to be bimodal, representing the “on” or “off” populations, respectively. A
740 cut-off to distinguish cells from each population could be set at a local minimum between these
741 two peak populations in the observed single cell expression distribution. To account for the
742 relatively low expression levels of transcription factors, we used the first local minimum closest
743 to the “off” population. An exception in this regard was made for *Hoxc4*, which was likely to be
744 in excess of bimodal, i.e., having at least three different expression levels of high, low and off. We
745 regarded the high and low *Hoxc4*$^{+}$ population as “on” and manually assigned the threshold, while
746 assuming the “low” and “off” peaks were proximal so that identifying a local minimum between
747 the two peaks was non-trivial.
748
749 To assign their regional identity, cells were categorized into Hoxc4$^{on}$, Hoxc4$^{on}$/Hoxa7$^{on}$,
750 Hoxc4$^{on}$/Hoxa7$^{on}$/Hoxc9$^{on}$, Hoxa7$^{on}$/Hoxc9$^{on}$, and Hoxc9$^{on}$ populations based on their binarized
751 expression. Within each of these regions, the Hoxa5$^{on}$, Hoxa5$^{on}$/Hoxc8$^{on}$ and Hoxc8 populations
752 were calculated as a ratio of the total number of cells. Scatter plots and bar charts were generated
753 using ggplot2 in R (Wickham, 2009).
754
755
756 **Analysis of Hox protein distribution in developing spinal cord**
757 Embryos from various developmental stages (E9.5–E12.5) were obtained from timed matings of
758 WT/*miR-23~27~24* DKO/*miR-196* DKO and *miR-196* TKO/Hoxc8:Cre;ROSA26-loxp-STOP-
759 loxp-tdTomato mice, and detection of a mating plug was counted as embryonic day 0.5 (E0.5).
760 Embryos were dissected out, fixed in 4% paraformaldehyde in PBS for 2 h, and balanced in 30%
761 sucrose after several washes. Fixed embryos were then embedded in OCT compound (Tissue-Tek),
762 frozen in dry ice, and stored at 80 ºC until use. Spinal sections (20 μm) were made with a CM 1950

763 cryostat (Leica) and immediately placed on slides. Based on the total number of sections and the
764 order of each segment from the spinal cord, the precise position of sections along the rostrocaudal
765 axis (from cervical to thoracic spinal cord) could be determined.
766
767 **Analysis of *Hoxa5* expression in embryoid bodies**
768 ESCs were cultured and differentiated into spinal MNs. Embryoid bodies (EBs) were harvested
769 after applying RA for 96 hours and fixing in 4% paraformaldehyde for cryosectioning. Different
770 kinds of Hoxa5 antibodies were used to perform immunocytochemistry and visualization of Hoxa5
771 expression was achieved using confocal imaging. The intensity of Hoxa5 protein signal was
772 analyzed using MetaXpress (Multi Wavelength Cell Scoring module, MWCS).
773
774 **Generation of inducible 'Tet-ON' ESCs**
775 Human HOXC8 and mouse Hoxa5 cDNAs were directionally inserted into pENTR/D-TOPO
776 vector (Life Technology) following manufacturer instructions. Primary miRNA sequence or
777 repetitive miRNA sponge sequence was synthesized and cloned into the 3' UTR of p2Lox-GFP.
778 Inducible lines were generated by treating the recipient ESCs for 16 h with doxycycline to induce
779 Cre, followed by electroporation of p2Lox-HOXC8:V5/Hoxa5/miRNA OE/miRNA SP plasmids.
780 After G418 selection, individual resistant clones were picked and characterized. After 10–15 days
781 of selection, clones were expanded. Details of primer and miRNA sequences are provided in SI
782 Table S5. Inducible miRNA overexpression and sponge ESCs were cloned into the 3' UTR of the
783 p2Lox-GFP construct, and the same procedure as described above was followed to generate stable
784 ESC clones.
785

## Statistical analyses and graphical representations

All statistical analyses were conducted using GraphPad Prism 6 (GraphPad Software). Values are presented as mean ± SD, as indicated. Student's *t*-tests were used for comparisons between experimental samples and controls. Statistical significance was defined as *p < 0.01 by Student's *t*-test.

## Mathematical models of mRNA-miRNA reaction networks

We described production, degradation and binding of mRNA ($R$) and miRNA ($r$) molecules with ordinary differential equations (ODEs) using mass action kinetics. Our mmi-1 -2 and -3 Models describe mRNA ($R$) containing one, two, or three binding sites for miRNA ($r$), respectively. Since binding and unbinding processes are much faster than production and degradation, we adopted a total quasi-steady-state assumption (Borghans *et al*, 1996) and reduced the ODEs with 3-5 state variables to only two ODEs describing slow processes and 1-3 algebraic equations describing fast processes. We analyzed the mmi-1 and mmi-2 Models using algebraic approaches to obtain conditions that allow three steady states, a necessary condition for bistability. The mmi-1 Model cannot be bistable for all parameters. For the mmi-2 Model, we obtained a relationship among parameters that allowed bistability. We confirmed that relationship using numerical methods, including simulations and numerical bifurcation analysis. For the mmi-3 Model, we used numerical approaches to find the range of parameters that allow bistability. Details of the mathematical analyses are included in the SI Text.

## Chemical Reaction Network Theory

We applied Chemical Reaction Network Theory (CRNT) (Feinberg, 2019) to assess the ability of the mmi-1 -2 and -3 Models to generate bistability. The mmi-1 Model is a deficiency zero system, so it is monostable for each combination of positive parameter values. The mmi-2 Model is a deficiency one system, so it is bistable for certain combinations of positive parameter values. Although CRNT analysis did not generate a definitive conclusion for the mmi-3 Model, its subnetwork contains the mmi-2 Model structure so it can also generate bistability for some combinations of positive parameter values (Conradi *et al*, 2007).

**Enumerating mRNA-miRNA reaction network motifs**

To estimate how frequently the mRNA-miRNA reaction network motif represented by the mmi-2 Model can be found in biological systems, we obtained a dataset on predicted miRNA binding sites in humans from TargetScan (Agarwal *et al.*, 2015). To estimate the lower bound of the number of incidences of the motif, we counted the number of mRNA-miRNA pairs in which the target mRNA has two or more conserved binding sites for the cognate miRNA. To estimate the upper bound, we counted the number of miRNA binding site pairs (conserved and non-conserved), each of which share a target mRNA and cognate miRNA.

**Spatiotemporal model for MN development**

We built spatiotemporal models for a group of developing MNs near the boundary between the Hoxa5- and Hoxc8-expressing regions. We modeled a grid containing 10×40 cells, where 40 is the number of cells along the rostrocaudal axis. In the models, diffuse RA and FGF molecules provide positional information along the rostrocaudal axis, which are produced at the rostral and

caudal boundaries of the domain, respectively. We assumed uniform degradation rate constants for RA and FGF in the domain, so the cells were influenced by exponential functions of RA and FGF gradient concentrations along the rostrocaudal axis in an antiparallel fashion. For each cell in the mmi-S Model, the spatiotemporal model describes the dynamics of Hoxa5 and Hoxc8 (mRNAs and proteins), as well as miR-27 and miR-196, under the influence of both RA and FGF. Key interactions of the GRNs include that: 1) RA activates Hoxa5 transcription (Li *et al.*, 2017; Liu *et al.*, 2001); 2) FGF activates Hoxc8 transcription (Liu *et al.*, 2001); 3) Hoxc8 inhibits Hoxa5 transcription (Dasen *et al.*, 2005; Philippidou & Dasen, 2013); 4) miR-27 inhibits Hoxa5 translation by binding to two or more sites at the 3' UTR (Li *et al.*, 2017); and 5) miR-196 inhibits Hoxc8 translation by binding to the 3' UTR (Wong *et al.*, 2015). We also assumed that miRNA-mRNA interactions occur at three predicted *miR-27* binding sites on *Hoxa5* mRNA and at four predicted *miR-196* binding sites on *Hoxc8* mRNA (as predicted by TargetScan) (Agarwal *et al.*, 2015), although only two of those binding sites in each mRNA-miRNA pair are essential for our conclusions. Influence diagrams of four alternative spatiotemporal models, i.e. T-CR, T-UR, Tmi-UR and Tmi-FB Models, are shown in Figures 2A, S2A, 4A, 4D respectively.

We performed numerical bifurcation analyses and simulations on these spatiotemporal models. The parameters values were chosen from randomly sampled sets and adjusted manually (see SI Text). Since mathematical analysis of the mmi-1, -2 and -3 Models revealed that a simple relationship among kinetic rates is sufficient to obtain switch-like behaviors, we chose parameter values for the mmi-S Model such that the Hoxa5$^{on}$ cells and Hoxc8$^{on}$ cells are differentiated by activation of two bistable switches governed by two pairs of mRNA-miRNA feedback loops, respectively. Accordingly, the qualitative behaviors of the model could be reproduced using a wide range of parameter values. Simulated cells were induced to express low levels of Hoxa5 and Hoxc8

before activation of RA and FGF signaling through morphogen production and diffusion. Steady-state distributions of Hoxa5$^{on}$ cells, Hoxc8$^{on}$ cells, and Hoxa5$^{on}$ Hoxc8$^{on}$ cells in terms of both protein and mRNA levels were analyzed and compared experimentally. *miR-27* and *miR-196* knockouts were simulated by turning off their productions in all cells. List of all models and their parameter values are included in SI Tables S1, S3 and S4.

## DATA AVAILABILITY

The datasets and computer code produced in this study are available in the following databases:

- Modeling computer scripts: GitHub (https://github.com/lfsc507/mmi)
- Single-cell RNA-sequencing data: Gene Expression Omnibus GSE156023 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE156023)

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## AUTHOR CONTRIBUTIONS

Conceptualization: J.A.C. and T.H.; Methodology: C.J.L., E.S.L. and T.H.; Modeling analysis: Z.L., A.W. and T.H.; Experiments: C.J.L., E.S.L. and J.A.C.; Resources: V.G. and E.G.; Writing – original draft: J.A.C. and T.H.; Writing – review & editing: J.A.C. and T.H.; Funding acquisition: J.A.C. and T.H.; Supervision: J.A.C. and T.H.

## DECLARATION OF INTERESTS

All authors declare no competing interests.

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