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The hybrid epithelial/mesenchymal phenotype and its implications in cancer metastasis

by

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ABSTRACT

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More than 90% of cancer-related deaths occur because cancer cells metastasize, i.e. invade the surrounding tissue, travel throughout the body, and form tumors at distant organs. Metastasis is often fueled by Epithelial-to-Mesenchymal Transition (EMT) that enables cells to migrate and invade, and its reverse Mesenchymal-to-Epithelial Transition (MET) that facilitates cells to shed migration and regain adhesion to colonize other organs. While undergoing EMT or MET, cells can adopt a hybrid epithelial/mesenchymal (E/M) phenotype through which they can both adhere and migrate, leading to collective migration as clusters of Circulating Tumor Cells (CTCs) that can be apoptosis-resistant and can initiate 50 times more tumors as compared to individually migrating CTCs. However, the hybrid E/M remains poorly characterized and has been tacitly assumed to be ‘metastable’ or transient. This study, through integrating mathematical modeling with wet-lab experiments, suggests that the hybrid E/M phenotype can be quite stable and its stability can aggravate tumor progression. First, we model the core regulatory network underlying EMT/MET – interconnected feedback loops among miR-34, miR-200, ZEB, SNAIL families – to predict that it can act as a ‘three-way’ switch enabling three phenotypes – epithelial (high miR-200, low ZEB), mesenchymal (low miR-200, high ZEB) and hybrid E/M (medium miR-200, medium ZEB). Second, GRHL2 and OVOL1/2 are predicted to stabilize a hybrid E/M phenotype and then confirmed experimentally in H1975 lung cancer cells that display a stable hybrid E/M phenotype. Third, modeling the interconnections of core EMT network with that regulating tumor-initiation potential (LIN28/let-7) predicts that a hybrid E/M, but not necessarily a fully mesenchymal,
phenotype associates with higher tumor-initiation potential. Finally, integrating the core EMT network with intercellular Notch signaling, we predict that Notch-Jagged signaling can give rise to clusters of cells in a hybrid E/M phenotype. This prediction corroborates with our experimental observations that the drug-resistant tumor-initiating cells display elevated levels of Notch-Jagged signaling, reflecting the metastatic potential of hybrid E/M cells that can form clusters of CTCs. These results strongly argue that cancer cells in a hybrid E/M phenotype can be the key ‘bad actors’ of metastasis and identify novel targets – OVOL1/2, GRHL2 and JAG1 – to curb metastatic load.
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<th>Description</th>
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<tbody>
<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
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<tr>
<td>MET</td>
<td>Mesenchymal to Epithelial Transition</td>
</tr>
<tr>
<td>CTC</td>
<td>Circulating Tumor Cells</td>
</tr>
<tr>
<td>DTC</td>
<td>Drug tolerant Cells</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer Stem Cells</td>
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<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<tr>
<td>HIF1α</td>
<td>Hypoxia-inducible growth factor 1 alpha</td>
</tr>
<tr>
<td>GRHL2</td>
<td>Grainyhead-like transcription factor 2</td>
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<tr>
<td>ZEB1/2</td>
<td>Zinc finger E-box binding homeobox 1/2</td>
</tr>
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<td>SIP1</td>
<td>Smad interacting protein 1</td>
</tr>
<tr>
<td>SNAI1/2</td>
<td>Snail Family zinc finger 1/2</td>
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<tr>
<td>TWIST1</td>
<td>Twist family BHLH transcription factor 1</td>
</tr>
<tr>
<td>FOXC2</td>
<td>Forkhead box protein C2</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>OCT4</td>
<td>Octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple Negative breast cancer</td>
</tr>
<tr>
<td>TEB</td>
<td>Terminal end bud</td>
</tr>
<tr>
<td>PSF</td>
<td>Phenotypic Stability Factor</td>
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Chapter 1

Introduction

1.1 Metastasis: an Achilles’ heel in cancer treatment

Since 1971 when President Nixon declared the ‘War on Cancer’, cancer research has witnessed remarkable progress in identifying the genetic mutations driving cancer initiation and progression, diagnosing cancer earlier, and deciphering the risk factors associated with developing cancer. However, cancer metastasis, the spread of cancer cells from the primary organ to other distant organs in the body and subsequent generation of secondary tumors, remains clinically insuperable (1) and accounts for more than 90% of all cancer-related deaths today (2).

During metastasis, a cancer cell usually executes the following steps: invade the surrounding tissue, enter blood and/or lymph vessels (intravasation), survive the treacherous journey during circulation while evading attacks by the immune system,
translocate to the vasculature of distant organs, exit circulation (extravasation), and finally adapt to the ‘foreign’ microenvironment of these organs to proliferate and form a macroscopic secondary tumor (colonization) (3, 4) (Figure 1.1). These steps are tremendously challenging and hence the rate of attrition during metastasis is huge (5); only <0.02% of the disseminated tumor cells (DTCs) are eventually successful in colonizing distant organs and form macroscopic secondary tumors or macrometastases (6). Therefore, while the malignant tumor cells may be shed off early in tumor progression, macrometastases might manifest clinically only during the later stages of the disease (5). Furthermore, DTCs can be present in the bone marrow of cancer patients for many years, but only 50% of them develop into metastases (7). Despite being such an inefficient process, metastasis remains the Achilles’ heel in cancer treatment. Therefore, elucidating the design principles underlying the metastatic cascade is of paramount importance.
Figure 1.1: Various steps of the metastatic cascade for (adenocarcinomas. EMT (Epithelial-to-Mesenchymal Transition) depicted by change of color of cells from blue to red) fuels the invasion of cells through the basement membrane (depicted in black) and intravasation. After exiting circulation at distant organs, carcinoma cells are believed to undergo MET to form secondary tumors (metastases) (4).

The abovementioned steps underlying the metastatic cascade do not hold true for sarcomas (cancers born in mesenchymal organs), or ‘liquid’ cancers such as leukemia, but are specific to carcinomas (cancers born in epithelial organs).

1.2 EMT/MET: the engine of cellular plasticity during metastasis

Various steps in the metastatic cascade entail cellular plasticity – the ability of a cell to acquire a new fate, often reversibly, upon exposure to certain conditions. An archetypical example of cellular plasticity during metastatic progression is the transition between epithelial and mesenchymal phenotypes (8, 9) – Epithelial to Mesenchymal Transition (EMT) and its reverse Mesenchymal to Epithelial Transition (MET). EMT usually marks the first step of metastatic cascade, enabling cancer cells to shed their cell-cell adhesion and apico-basal polarity and gain the ability to migrate and invade basement membrane, finally entering the circulation. MET, on the other hand, is believed to support the last step of this cascade. It enables the cells that exit circulation to arrive at distant organs to shed their migratory and invasive traits and regain cell-cell adhesion to initiate a tumor (10). Notably, EMT and MET are not unique to cancer; in fact, they are key physiological processes that are crucial during embryonic development and tissue repair (11, 12).
While EMT and MET are largely accepted as fundamental steps in embryonic development (11), their functional contribution to metastasis has, however, been called in question. The initial arguments against recognizing the contribution of EMT to metastasis was driven by challenges in identifying migratory cancer cells. However, these have been largely countered with subsequent observations enabled by powerful imaging and lineage-tracing techniques showing that cancer cells at the invasive front often have compromised cell-cell junctions and E-cadherin levels and a gain of mesenchymal markers (13), EMT facilitates more aggressive behavior in both mouse and human carcinoma models (13, 14), and the expression of mesenchymal markers on migratory and invading cells (10, 15, 16). The presence of EMT-related markers on circulating tumor cells (CTCs) in multiple cancer types (17–19) has further consolidated the role of EMT in fueling metastasis.

Lately, however, the role of EMT in metastasis has been confounded largely due to a tacit assumption to view EMT as an ‘all-or-none’ or a binary process. Consequently, although EMT has been categorized as type I (developmental EMT), type II (wound healing/tissue repair EMT) and type III (EMT during metastasis)(20), the concept that EMT might refer to a different set of changes in cell behavior and/or lineage depending on the biological context remains under appreciated.

EMT was initially described as ‘epithelial-mesenchymal transformation’ by Elizabeth Hay in her pioneering work on primitive streak formation in the chick, where it referred to a switching of the developmental lineage from epithelium to mesenchyme (21). However, such a ‘transformation’ is not necessarily, if at all, involved during tumor
progression (22, 23). Meanwhile, the term ‘transformation’ has given way to ‘transition’ to reflect the accumulating evidence that EMT and neoplastic transformation are quite different processes and that unlike many lineage-commitment decisions, EMT is reversible (9).

Moreover, recently increasing acknowledgement that EMT need not be an ‘all-or-none’ process and that cells can exhibit a spectrum of intermediate states in between epithelial and mesenchymal (8, 9, 18, 19, 24, 25) has emerged as a compelling alternative explanation (10, 26) for recent studies (27, 28) that claim that EMT is dispensable for metastasis. Besides, the concept of an intermediate or a partial EMT or a hybrid epithelial/mesenchymal (E/M) phenotype has also been invoked to reconcile the paradox that despite a proposed role of EMT in metastasis, most metastatic carcinomas exhibit epithelial well-differentiated characteristics (29). Overall, the consideration of EMT as a binary process has significantly undermined both the underlying fine-tuned plasticity of cancer cells and its implications in fueling metastasis, thereby obfuscating our understanding of the mechanistic underpinnings of metastasis.

1.3 Hybrid E/M or partial EMT phenotype

The recognition about EMT not being an ‘all-or-none’ process has existed in the context of wound healing (30, 31) and morphogenesis (32) for almost a decade, and has since gained further attention in the contexts of mammary morphogenesis, gastrulation, and neural crest migration (33–37). However, the evidence that EMT and MET are not binary, but instead multi-state processes wherein cells can attain intermediate
phenotype(s) between pure epithelial and pure mesenchymal, has been gathered relatively recently in cancer, largely during the course of this thesis. These intermediate phenotypes, the number of which still remain to be resolved, have been often interchangeably referred to as ‘partial EMT’, ‘partial Mesenchymal-Epithelial reverting transition (MErT)’, ‘EMT-like’, ‘incomplete EMT’, ‘hybrid E/M phenotype’, ‘intermediate EMT’, ‘intermediate epithelial’, ‘intermediate mesenchymal’, ‘semi-mesenchymal’, and ‘reversed epithelia’ (32, 37–57). Despite different semantics, all these observations point to some degree of co-expression of epithelial and mesenchymal markers, and often an amalgamation of epithelial and mesenchymal traits such as coupled adhesion and migration to enable collective cell migration (Figure 1.2).

Cells expressing both epithelial and mesenchymal markers have been found in the bloodstream of breast, lung, colon, and prostate cancer patients (17–19, 58, 59). Similar observations have been made in cell lines belonging to breast, ovarian, lung, and renal cell carcinoma (38, 39, 42–44), primary breast and ovarian cancer cells (18, 60), metastatic brain tumors (49), as well as in many mouse models of pancreatic ductal adenocarcinoma (PDAC) and prostate cancer (61, 62). Moreover, recent evidence in kidney fibrosis also indicates a partial instead of a full EMT (63, 64).

Alarmingly, cells co-expressing epithelial and mesenchymal markers are most enriched in the breast cancer subtype with poor clinical outcome – triple-negative breast cancer (TNBC) (18). Consistently, in aggressive cancers such as basal-like breast cancer (BLBC) and melanomas, co-expression of epithelial markers such as cytokeratins 8 and 18 and mesenchymal marker such as vimentin, instead of the expression of vimentin
alone, correlates with enhanced metastatic potential and poor survival (65–69). Moreover, combined expression of E-cadherin and fibronectin (an EMT biomarker (70)) correlates with high histologic grade, lymphovascular invasion, and triple negativity, and is an independent prognostic factor in patients with invasive breast carcinoma(71). Also, lung cancer patients co-expressing an EMT inducer SNAI2 and the epithelial protein CDH1 had significantly reduced survival (43). Put together, these observations strongly suggest that a hybrid E/M phenotype can be considered to be the hallmark of tumor aggressiveness.

Figure 1.2: Canonical morphological and functional characteristics of phenotypes – epithelial (E), hybrid epithelial/mesenchymal (E/M), and mesenchymal (M) (9).

1.4 Hybrid E/M phenotype and clusters of Circulating Tumor Cells

A partial EMT phenotype has been well-studied in the context of embryonic development, especially the branching morphogenesis of the mammary gland (33–37, 51). During branching morphogenesis, tubular epithelial structures split repeatedly to generate a ductal tree; and the ‘tip’ cells located at the cap of terminal end buds (TEBs) of the growing tubule display partial EMT features. These cells adhere to their neighbors as well as transiently lose their apico-basal polarity and migrate in response to
extracellular signals (33, 72). Similarly, in wound healing, immature keratinocytes at the edge of wound migrate collectively and re-epithelialize to close the wound (31, 73). Such collective migration offers multiple advantages to cells such as allowing mechanical coupling among them, maximum plasticity to switch to either epithelial or mesenchymal phenotypes, and enabling cells to migrate due to detection of external signals made by their neighbors (72). These advantages can be crucial during invasion and intravasation of multicellular strands of carcinoma cells (74).

Collective migration of cancer cells has also been observed both in mouse models and human samples. Recent analysis by Bronsrt and colleagues of the 3D reconstruction from 2D serial sections of multiple tumor types – colorectal cancer (CRC), liver metastases of CRC, PDAC, lung adenocarcinoma, and invasive ductal breast carcinoma – elucidated ‘tumor buds’ that are detached from the main tumor mass mostly as clusters of <5 cells. They found no evidence of single-cell migration and suggested that the single-cell migration of cancer cells is very rare (<0.003% of total tumor cell number), and therefore, cancer cells migrate mostly collectively (75). Similarly, circulating tumor cells (CTCs) moving as clusters have been observed in melanoma, prostate, lung and breast cancer patients (76–78) and mouse models of breast cancer (76, 79). Both studies using mouse models highlight the heightened metastatic potential of these clusters. Aceto et al. (76) demonstrate that although clusters constitute a tiny fraction of total CTC events (~3%), they contribute 50% of total metastases, whereas Cheung et al. (79) claim that over 97% of metastases were formed by clusters. These clusters are usually 2-8 cells large, resistant to apoptosis, are formed before entering the circulation (76, 79), and are more likely to be trapped in blood vessels for intravasation and extravasation (80, 81).
These traits offer a potential mechanistic understanding for why the presence of these clusters or emboli in patients has been consistently reported to strongly correlate with poor prognosis in cancer patients, even before the phenomenon of EMT was characterized (78, 82, 83).

Figure 1.3: Enhanced metastatic potential of clusters of Circulating Tumor Cells (CTCs). Although most CTCs released from the primary tumor are single cells (red), some CTC clusters are also shed (blue). These clusters have high levels of plakoglobin and have an enhanced potential for forming lung metastases (84).

The molecular characterization of both the tumor buds and clusters of CTCs further strengthen the link between a hybrid E/M phenotype and the migration of clusters of CTCs. Tumor buds analyzed by Bronsert and colleagues co-expressed an epithelial protein E-cadherin and an EMT-inducing transcription factor ZEB1 (75). Other tumor buds also mirrored the staining pattern (85). Similarly, the CTC clusters retained their epithelial characteristics while gaining the expression of certain genes usually associated
with a mesenchymal trait (77, 79). However, CTC clusters need not necessarily contain only hybrid E/M cells; some of these clusters may contain leukocytes, platelets, and endothelial cells that may help evade immune attack while in circulation (18, 86, 87).

1.5 Hybrid E/M phenotype and tumor-initiation potential

Another aspect where the categorization of EMT as a binary process has, at least in part, confounded our understanding is the association between EMT and tumor-initiation potential (9, 88). Initially, immortalized human mammary epithelial cells undergoing a full EMT were proposed to gain ‘stemness’ – the ability to self-renew as well as differentiate into other types, eventually giving rise to a tumor clone in the long-term – and behave operationally as tumor-initiating cells (89, 90). Similar findings were reported for pancreatic, colorectal, and hepatocellular carcinomas (4). Therefore, aberrant activation of EMT was believed to serve two complementary roles during metastatic progression – (a) fuel the invasive and migratory traits of cells to enable translocation to distant organs, and (b) facilitate tumor initiation at the secondary/metastatic site upon arrival (88). However, this notion of a full EMT coupled with ‘stemness’ has been recently challenged by studies showing that repression of EMT is essential for effective secondary tumor-initiation (91–94). A few studies have attempted to resolve this contradiction by suggesting that the cells in a hybrid E/M phenotype are most likely to gain stemness. Most of these studies have been published during the course of this thesis, strongly corroborate with the predictions of mathematical models presented in this thesis, and are therefore discussed in detail in Chapter 3.
1.6 Scope of thesis

The aim of the work in this thesis is to characterize a hybrid epithelial/mesenchymal (E/M) and its implications in cancer metastasis via an integrated theoretical and experimental approach. A hybrid E/M or partial EMT phenotype has been tacitly assumed to be ‘metastable’ or transient, i.e. cells cannot maintain it for a long time (95). This thesis investigated the proposed ‘metastability’ from a nonlinear dynamics perspective.

Chapter 2 focuses on mathematical modeling of the core EMT/MET regulatory network that comprises two interconnected mutually inhibitory feedback loops – each between an EMT-inhibiting microRNA family and an EMT-inducing transcription factor family – (miR-34/SNAIL) and (miR-200/ZEB). A new theoretical framework that captures quantitatively the microRNA-mediated regulation is developed(96). The model predicts that this core network can behave as a ‘three-way switch’ leading to three stable steady states (phenotypes) – epithelial (E), mesenchymal (M), and hybrid E/M. Under certain conditions, cells in different phenotypes can switch among one another, i.e. more than one phenotypes can co-exist (97, 98). The set of existing experimental observations that the model can explain is also discussed.

Chapter 3 focuses on identifying ‘phenotypic stability factors’ for a hybrid E/M phenotype based on integrated mathematical modeling and experiments. H1975 lung cancer cells are presented as an example of a stable hybrid E/M phenotype, and OVOL1/2 and GRHL2 are proposed as a set of players contributing to the stability of the
hybrid E/M phenotype. Knockdown of these players drives a complete EMT in H1975 cells as predicted by the mathematical model (99, 100).

Chapter 4 focuses on mathematical modeling of the interconnections between the core EMT /MET regulatory network with the stemness decision-making loop between LIN28 and let-7. The model predicts that although both hybrid E/M and a fully mesenchymal phenotype can gain stemness or tumor-initiation potential, a hybrid E/M phenotype is more likely to gain stemness as compared to a fully mesenchymal phenotype, specially under conditions of high inflammation (101). In other words, the ‘stemness window’ lies midway on the ‘EMT axis’ with E and M as the two ends.

Chapter 5 focuses on modeling the intracellular core EMT network with the intercellular Notch signaling. We predict that Notch-Jagged signaling can give rise to clusters of cells in a hybrid E/M phenotype. Further, we present preliminary experimental data showing that drug-resistant cells display enhanced levels of Notch-Jagged signaling, thereby validating our hypothesis about the role of (stable) hybrid E/M phenotype in drug resistance and/or tumor-initiation(102).

Chapter 6 summarizes the key findings in the thesis.
Tristability in the core EMT/MET network

2.1. Introduction: The core EMT/MET regulatory network

Epithelial cells can undergo a partial or complete EMT in response to a multitude of signaling pathways such as TGFβ (transforming growth factor beta), HGF (hepatocyte growth factor), EGF (epidermal growth factor), HIF1α (hypoxia-inducible growth factor 1 alpha), Notch, FGF (fibroblast growth factor), Wnt and IGF (insulin-like growth factor) (103) and extracellular mechanical factors such as ECM (extracellular matrix) density (104, 105). These signals often activate one or more of the pleiotropic EMT-inducing transcription factors (EMT-TFs) – TWIST1 (Twist family BHLH transcription factor 1), SNAI1/2 (Snail family zinc finger 1/2), ZEB1 (zinc-finger E-box binding homeobox 1; also referred to as δEF1 – Delta-crystallin enhancer binding factor 1), ZEB2 (also referred to as SIP1 – Smad-interacting protein 1), Goosecoid, FOXC2 (forkhead box C2), and PRRX1 (paired-related homeobox 1) – that directly or indirectly repress E-cadherin.
The inhibition of E-cadherin and its delocalization from the membrane are considered to be the hallmarks of EMT that often lead to reduced cell-cell adhesion and increased migratory traits. On the other hand, EMT can be inhibited by MET-inducing transcription factors (TFs) such as GRHL2 (grainyhead-like transcription factor 2), OVOL1/2 (ovo-like zinc finger 1/2), and ELF5 (E74-like ETS transcription factor 5), and p53 that can activate EMT-inhibiting microRNA (miR) families miR-200 and miR-34 and inhibit EMT-TFs, therefore restoring E-cadherin levels and/or localization (35, 103, 106–111). In addition to inhibition of E-cadherin, EMT is often characterized by increase of mesenchymal markers such as N-cadherin and intermediate filament vimentin(103).

In many carcinomas, the abovementioned signals converge on a core regulatory network of EMT/MET that comprises of two miR families miR-34 and miR-200 and two TF families SNAIL and ZEB (112, 113). This core network is referred to as ‘motor of cellular plasticity’ due to its coupling with other cellular properties such as cell cycle, metabolism, angiogenesis, cell-cell communication, stemness or tumor-initiation potential, multidrug resistance, immunosuppression and apoptosis (98, 113–116), many of which constitute the ‘hallmarks of cancer’(117).

The components of this core network form two mutually inhibitory feedback loops – one between miR-34 and SNAIL, and the other between miR-200 and ZEB (112, 118–120). Further, these feedback loops are interconnected – SNAIL, a self-inhibiting EMT-TF (121), can activate ZEB (122) and inhibit miR-200(119), and ZEB, a self-activating EMT-TF (123, 124), can inhibit miR-34 (125). An epithelial phenotype
corresponds to high levels of miR-34 and miR-200, whereas mesenchymal phenotype is characterized by high levels of ZEB and SNAIL (Figure 2.1).

The aim of this study was to elucidate whether this core network can have three stable steady states (phenotypes), and if yes, predict the expression profile of the hybrid E/M phenotype.

**Figure 2.1: Core EMT network and its connection with other cellular traits.** Core EMT network (shown in yellow box) consists of two interconnected mutually inhibitory feedback loops – (miR-34/SNAIL and miR-200/ZEB). Solid bars represent transcriptional inhibition, solid arrows denote transcriptional activation, and dotted lines denote miRNA-mediated regulation. Numbers mentioned alongside each regulation are the number of binding sites experimentally determined or estimated. This core network receives inputs from a variety of signals (shown by I), modulates many cytoskeletal elements (E-cadherin, N-cadherin, vimentin) and couples with many other cellular traits, thereby being referred to as the ‘motor of cellular plasticity’ (9).
2.2 Theoretical framework to study microRNA-based regulation

Mutually inhibitory loops between two fate-determining TFs usually form the cornerstone of many binary cell-fate decisions (126). For instance, cross-inhibitory PU.1 and GATA.1 regulate the decision between erythroid and myeloid lineages in hematopoiesis, and mutually inhibiting OCT4 and CDX2 control the decision between ‘sister’ fates - trophoectoderm and inner cell mass. Mutual inhibition between the two TFs A and B, therefore, often leads to mutual exclusivity of the expression of A and B, and consequently the two cell-fate identities (126). Such feedback loops are well-studied theoretically and experimentally, and usually behave as binary switches enabling two stable states – (high A, low B) and (low A, high B) – each corresponding to a cell-fate (127–132). It has also been proposed that if one or both of the TFs self-activate themselves, a third stable state can exist – (medium A, medium B) – that can act as the ‘poised’ state of a progenitor cell capable of differentiating into either lineage (128, 129).

However, the core EMT network included chimeric transcriptional-translational feedback loops. Hence, to capture the different dynamics of transcriptional regulation vs. microRNA-based translational regulation (133–135), as well as incorporate the effect of binding of different number of binding sites of a miR on a mRNA (136, 137), we developed a novel theoretical framework to represent microRNA-mediated regulation (96) (Figure 2.2). MicroRNAs can inhibit translation of their target proteins by degrading their mRNAs and/or sequestering the mRNA to prevent translation, and can get degraded or recycled in the process too (138). All these mechanisms are captured in our framework by considering the chemical binding/unbinding reactions between miR and mRNAs.
In this framework, one or more miR (μ) molecules can bind reversibly to the 3’ UTR of a target mRNA (m) molecule to form a microRNA-mRNA complex (μ-m). Consider a mRNA with n possible sites for the binding of miR; there are (n+1) possible configurations of the mRNA — ranging from no miR bound to it to all n sites being occupied by miR. The binding of different miR molecules is considered to be independent of each other, because a miR is only 22 nucleotide (nt) long and recognizes mRNA by a seed sequence of 7-8 nt(139). Let us consider the binding rate of a miR molecule to a mRNA molecule as $r_{μ⁺}$, and unbinding rate as $r_{μ⁻}$. At equilibrium, the concentration $[m_i]$, i.e. the mRNA molecule to which i molecules of miR are bound, obeys equation (1):

$$r_{μ⁺}[m_i] = r_{μ⁻}[m_{i+1}]$$

Let us refer to the ratio of binding and unbinding rates $r_{μ⁺} / r_{μ⁻}$ as $μ_0$. Hence, $[m_i] = [m_0](μ / μ_0)^i$. For the case of $m_i$, $i$ out of $n$ binding sites can be occupied by miR in $C_n^i = \frac{n!}{i!(n-i)!}$ number of ways. The total concentration of mRNA is split across multiple such configurations, each with a different number of binding sites occupied. This condition is mathematically represented as $\sum_{i=0}^{n} C_n^i [m_i] = m$.

Consequently, $m_i$ can be represented as a fraction of m by equation 2:

$$[m_i] = mM_n^i(μ) = m\frac{(μ / μ_0)^i}{(1 + μ / μ_0)^n}$$
Therefore, the total translation rate is given by:

$$
\sum_{i=0}^{n} l_i C_n^i [m_i] = m \sum_{i=0}^{n} l_i C_n^i M_n^i (\mu) = mL(\mu) \quad \ldots \ldots \ldots (3)
$$

Total active mRNA degradation rate is given by:

$$
\sum_{i=0}^{n} \gamma_m C_n^i [m_i] = m \sum_{i=0}^{n} \gamma_m C_n^i M_n^i (\mu) = mY_m (\mu) \quad \ldots \ldots \ldots (4)
$$

Total active miR degradation rate is given by:

$$
\sum_{i=0}^{n} \gamma_{m\mu} C_n^i [m_i] = m \sum_{i=0}^{n} \gamma_{m\mu} C_n^i M_n^i (\mu) = mY_{m\mu} (\mu) \quad \ldots \ldots \ldots (5)
$$

The typical form of these functions is shown in Figure 2.2, and the detailed derivation of these rates is presented in Appendix 1, Figure S1.1.

Hence, the generic deterministic equations for a chimeric feedback loop between microRNA ($\mu$) that targets the mRNA ($m$) of a transcription factor $B$ are given by:

$$
\frac{d\mu}{dt} = g_{\mu} - mY_{\mu}(\mu) - k_{\mu}\mu \ldots \ldots \ldots (6)
$$

$$
\frac{dm}{dt} = g_{m} - mY_{m}(\mu) - k_{m}m \ldots \ldots \ldots (7)
$$

$$
\frac{dB}{dt} = g_B mL(\mu) - k_B B \ldots \ldots \ldots (8)
$$

where $g_{\mu}$ and $g_{m}$ represent the synthesis rate of miR and mRNA, $g_B$ denotes the translation rate of mRNA into protein in absence of miR; $k_{\mu}, k_{m}$, and $k_B$ denote corresponding degradation rates.
As miRNAs are typically more stable than mRNAs with their half-lives an order of magnitude less than those of mRNAs (140–142), the three-equation system presented above can be reduced to a two-equation system assuming \( m \) reaches steady states. Using this theoretical framework and capturing the quantitative details known experimentally such as number of binding sites of miR-34 on SNAIL mRNA and those of miR-200 on ZEB mRNA (Figure S1.5), deterministic equations for (miR-34/SNAIL) standalone circuit driven by an external signal I, (miR-200/ZEB) standalone circuit driven by SNAIL, and those for the combined (miR-34/ SNAIL/miR-200/ZEB) circuit driven by I can be derived and are presented in Appendix 1.
Figure 2.2: The microRNA-based circuit (MBC) modeling approach. (A) Schematic diagram for the MBC model. One or more microRNAs ($\mu$) reversibly bind to mRNA ($m$) to form a $\mu$–$m$ complex. $k_m$ and $k_\mu$ are innate degradation rates of $\mu$ and $m$, respectively. $\mu$ can inhibit the translation of $m$ [the translation rate is scaled by $L(\mu)$] and degrade mRNA actively [at rate $Y_m(\mu)$]. The combined silencing effects can be characterized by $P(\mu)$. Also, $\mu$ can itself be degraded actively [at rate $mY_\mu(\mu)$ for $\mu$ microRNAs]. (B) The values of the $L$, $Y_m$, $Y_\mu$ as the functions of $\mu$ (scaled by the threshold $\mu_0$). The number of microRNA binding sites on the mRNA is taken to be six. A vertical dotted line is plotted to show the values at $\mu_0$. (C) The values of the shifted Hill function versus TF levels (scaled by the threshold $TF_0$). Here, the Hill coefficient is three, and the fold change $\lambda$ is 10.

2.3 Results

2.3.1 miR-34/SNAIL behaves as a noise-buffering integrator

To mimic the response of standalone (miR-34/SNAIL) circuit to a variety of external signals such as hypoxia (143), the typical phase space diagram of the circuit was plotted (Figure 2.3A, S1.3). As can be observed, only a single steady state exists corresponding to fixed values of SNAIL and miR-34. Modulating the value of $I$ smoothly alters these fixed values but does not lead to the existence of another steady state, indicating that standalone (miR-34/SNAIL) circuit cannot give rise to phenotypic transitions. Furthermore, the self-inhibition on SNAIL was shown to reduce or buffer the effect of external noise in any incoming signal on the core circuit (Figure S1.6), thereby preventing any aberrant activation of EMT due to transient or weak signals, and contributes to stably maintain an epithelial phenotype (144).
Figure 2.3: Dynamical system characteristics of miR-200/ZEB and miR-34/SNAIL modules. The figures show the nullclines and the possible states in the phase-spaces corresponding to the two modules (details of equations in Appendix 1). (A) The miR-200/ZEB driven by signal SNAIL is tristable. Red nullcline is for \( d\mu_{200}/dt = 0 \) and \( dZ/dt = 0 \), and blue nullcline is for the condition \( dmZ/dt = 0 \) and \( dZ/dt = 0 \). (B) The miR-34/SNAIL is monostable. Blue nullcline is for the conditions \( d\mu_{34}/dt = 0 \) and \( dS/dt = 0 \), and red for \( dmS/dt = 0 \) and \( dS/dt = 0 \). The variables \( \mu_{200}, \mu_{34}, m_Z, m_S, Z, \) and \( S \) correspond to the levels of miR-200, miR-24, ZEB mRNA, SNAIL mRNA, ZEB, and SNAIL, respectively. Green solid dots denote stable fixed points, and green unfilled circles denote unstable fixed points. The value of the SNAIL signal for A is taken from the stable fixed point in B. K Molecules, thousand molecules.

2.3.2 miR-200/ZEB behaves as a ‘three-way’ switch

Next, (miR-200/ZEB) standalone circuit driven by SNAIL (activation of ZEB and inhibition of miR-200) was analyzed. A typical phase space diagram (Figure 2.3B) indicates that three stable states can co-exist – (high miR-200, low ZEB), (low miR-200, high ZEB), and (medium miR-200, medium ZEB) denoted as \((1, 0)\), \((0, 1)\) and \((\frac{1}{2}, \frac{1}{2})\) respectively. The \((1, 0)\) and \((0, 1)\) states correspond to epithelial and mesenchymal phenotypes respectively as noted experimentally (119), whereas the \((\frac{1}{2}, \frac{1}{2})\) state was proposed to be associated with a hybrid E/M phenotype. A recent study indicating that
ZEB1 and miR-200 levels are different in cell lines categorized as epithelial, mesenchymal, or ‘undefined’ (i.e. hybrid E/M) in the NCI-60 cohort validates the association of hybrid E/M phenotype with a (medium miR-200, medium ZEB) (145).

2.3.3 Bifurcation diagram shows phases of co-existing phenotypes

Next, we analyzed the response of the (miR-200/ZEB) feedback loop for varying values of SNAIL. A typical bifurcation diagram shows the regions of coexistence of and transitions among multiple steady states. Five such phases are identified – two monostable ones \{E\} and \{M\}, two bistable ones \{E, M\} and \{E/M, M\}, and one tristable one \{E, E/M, M\}, where each phase represents the set of different phenotypes that can co-exist and transition among the states observed in that phase (Figure 2.4A). Thus, at low SNAIL levels, cells can adopt either an E or a hybrid E/M state; consequently, cells with identical genetic background can be expected to resolve into two subpopulations that can be resolved via FACS.
**Figure 2.4: Bifurcation and phase-diagram of the driven miR-200/ZEB decision module.** (A) Bifurcation of ZEB mRNA levels when driven by a signal S representing SNAIL. The bifurcation illustrates the possible coexistence (for some range of S) of three states: (i) the (1,0) state with high miR-200 and low ZEB, which corresponds to the epithelial (E) phenotype; (ii) the (0,1) state, which corresponds to the mesenchymal phenotype (M); (iii) the (½, ½) state, which corresponds to the hybrid phenotype (E/M). Starting with the (1,0) state (E) and increasing SNAIL, the circuit undergoes a transition to the (½, ½) state (E/M)—the first upward arrows on the right. Further increase in SNAIL leads to a transition from the (½, ½) state to the (0, 1) state (M)—the second upward arrows on the right. Starting from the (0, 1) state and decreasing SNAIL yields a direct transition to the (1,0) state—the downward arrows further to the left. (B) The phase-diagram of the chimeric circuit when it is driven by two independent signals S1 and S2, as is illustrated in the Inset circuit. Each phase corresponds to a different combination of coexisting states. For example, in phase {E}, only state (1,0) is stable, and, in phase {E,E/M}, the states (1,0) and (½, ½) can coexist.

Further, the action of SNAIL is represented by two independent signals – S₁ (transcriptional inhibitor of miR-200) and S₂ (transcriptional activator of ZEB), to represent the condition that SNAIL activity may depend on different cofactors and/or its post-translational modification. The resulting phase diagram illustrates two additional phases than those observed in the bifurcation diagram – monostable {E/M} phase and bistable {E, E/M} phase (Figure 2.4B), hence consolidating the proposition of a hybrid E/M state as a stable phenotype that cells can maintain. The richness of these identified phases may help explain why the extent of induction of EMT by SNAIL might vary – such as a full EMT in mesoderm of early chick embryo, no EMT in migration of axial mesendoderm (146), and partial EMT in response to hypoxia in breast cancer cells(143).
2.3.4 Dynamics of the combined core network

The combined core circuit includes inhibitory feedback from ZEB to miR-34. Upon including this feedback inhibition, the combined core network retains its tristable nature, as shown by bifurcation of ZEB mRNA levels in response to an external signal I that activates SNAIL (Figure 2.5A). To investigate the possible functional consequences of this inhibition of miR-34 by ZEB, the strength of this inhibitory link was varied. A stronger inhibition shifted the bifurcation curve further to the left, thereby introducing a stronger asymmetry in EMT and MET: whereas EMT proceeded via a hybrid E/M phenotype, MET proceeded directly from mesenchymal phenotype to an epithelial one (Figure 2.5B). On the contrary, replacing the inhibitory link from ZEB to miR-34 with a hypothetical activation link enabled MET also to proceed via attaining a stable E/M phenotype en route.

Further, to explore the contribution of ZEB self-activation in the tristability of the core network, this self-activation was deleted or made non-cooperative (i.e. a Hill function of rank 1). In either case, the hybrid E/M state was not observed (Figure S1.4), both for the case of standalone (miR-200/ZEB) and the combined core network, indicating that cooperative self-activation of ZEB is necessary for attaining the hybrid E/M phenotype. Similarly, the model predicted that both translational repression and mRNA degradation, with relatively greater contribution by the translational repression, by miR-200 are required for the existence of a hybrid E/M phenotype (Figure S1.2).
Figure 2.5: The phase-space and bifurcation diagram of the core regulatory unit. (A) The nullclines and the possible states in the phase-space corresponding to the combined regulatory unit (equations given in Appendix 1). The blue nullcline is for the whole circuit when all ODEs are set to 0 except for dmZ/dt = 0 (red line), and the red nullcline is for the conditions when all ODEs are set to 0 except for dµ200/dt = 0. Green dots denote stable fixed points, and green unfilled circles denote unstable/saddle fixed points. (B) Bifurcation plots for ZEB levels with respect to driving signal I. Different lines show different cases of feedback from ZEB to miR-34 (regulation fold change is denoted by $\lambda_{Z,\mu34}$). Compared with case without feedback (black, $\lambda_{Z,\mu34} = 1$), strong (red, $\lambda_{Z,\mu34} = 0.2$) or weaker repression (brown, $\lambda_{Z,\mu34} = 0.5$) makes bifurcation slightly shift to the left whereas activation (blue, $\lambda_{Z,\mu34} = 2.0$) makes it shift mainly to the right. Vertical dotted lines show the transitions among different states along the bifurcation curves.
2.3.5 Asymmetry in EMT/MET dynamics

To characterize the predicted asymmetry in EMT and MET, we applied a temporally varying signal I to the core EMT regulatory network (Figure 2.6A). It activates SNAIL and the cells are in an epithelial state initially. We superimposed the dynamics of ZEB levels on the bifurcation of ZEB levels in response to varying SNAIL (Figure 2.6B). Cells transition from being epithelial to hybrid E/M as evident with the increase in levels of ZEB mRNA and ZEB protein concomitantly with decrease in miR-200 levels, before undergoing another transition from hybrid E/M to being fully mesenchymal with further increase in signal. In contrast, as observed in the bifurcation diagram (Figure 2.4A), a transition from mesenchymal phenotype to the epithelial phenotype is direct, as shown in the deterministic simulation (Figure 2.6C-E). However, under the effects of cellular noise, mesenchymal to hybrid E/M transitions can also occur.

We also noted another aspect of the asymmetry between EMT and MET. Transition from epithelial to a hybrid E/M phenotype is observed when the input signal crosses the threshold amount of it required to trigger SNAIL levels, thereby increasing ZEB levels consequently (Figure 2.6A, B). Upon completion of EMT, ZEB levels remain high (Figure 2.6C) implying that cells can maintain high levels of ZEB (potentially through a direct or indirect self-activation), in agreement with the experimental observations (147). However, when the input signal is brought back to its basal levels (i.e. threshold amount of it to activate SNAIL), the cells do not switch back to being epithelial, and the signal has to be reduced much lower than its basal levels to induce a MET (Figure 2.6A-C). In other words, lifting the activation on SNAIL is insufficient to
induce MET; rather, SNAIL levels needs to be inhibited significantly to trigger MET. This prediction of the model resonates with experimental observations that activation of miR-34 is required to trigger MET(137).

Figure 2.6: Temporal dynamics of the complete and partial epithelial–hybrid–mesenchymal transition. (A) The effect of time-varying external signal. The basal signal level is shown in a purple dotted line. The input signal starts from zero (blue) at day 0 and increases to 100 K molecules (green) at day 25. Then, the input signal linearly decreases until day 50 (red). (B) The dynamics of ZEB in response to the input signal is shown in the SNAIL-ZEB phase-plane. The colors for the signal and the
corresponding response are matched. Both A and B also include arrows to clarify the time evolution. A bifurcation plot for ZEB with respect to SNAIL (black) is superimposed. (C) Temporal evolution of miR-200 (navy), ZEB mRNA (red), and ZEB protein (blue, scaled by 0.33 to fit in the plot). In A–C, we labeled the states at the basal signal with red dots. The plot shows that cells at the basal signal level are epithelial during EMT but are still mesenchymal during MET. Thus, the two-way transitions are dynamically asymmetric. The areas marked in gray are expanded in D to show that cells going from an epithelial to a mesenchymal state pass through a partial EMT state with intermediate levels of miR-200 and ZEB. (E) but cells directly return from a mesenchymal to an epithelial state without going through any intermediate state.

2.4 Discussion

Here, we introduced a novel theoretical framework (96) to incorporate the involvement of microRNAs in cell-fate decision making. This framework focuses on the binding/unbinding between mRNA and microRNA, captures both translational repression and active degradation of the miR-mRNA complex, and illustrates how the different number of binding sites of microRNA on an mRNA can alter the nonlinearity and dynamics of a chimeric TF-miR mutually inhibitory feedback loop. This framework elucidated the modular design principles of the core regulatory network governing EMT and MET. More specifically, the model predicted that while the standalone (miR-34/SNAIL) feedback loop acts as a noise-buffering integrator, the (miR-200/ZEB) feedback loop behaves as a ‘three-way’ switch enabling the co-existence of and transitions among the epithelial (high miR-200, low ZEB), mesenchymal (low miR-200, high ZEB) and hybrid E/M (medium miR-200, medium ZEB) phenotypes (97, 98).

These different proposed roles of (miR-200/ZEB) and (miR-34/SNAIL) offer a conceptual paradigm to unite several experimental observations – (a) SNAIL initiates the
repression of E-cadherin and ZEB1 is crucial for its significant repression that triggers an EMT (148), (b) complete MET requires a knockdown of ZEB1 (149) and that knockdown of SNAIL might not be sufficient, (c) cells treated with TGF-β to induce an EMT do not revert to an epithelial phenotype if their ZEB levels are high but can revert for sufficiently low levels of ZEB, suggesting that (miR-200/ZEB) marks the commitment point for cells undergoing an EMT (147), and (d) loss of ZEB1 protein can be sufficient to revert an EMT (150). Furthermore, the different levels of ZEB1 and miR-200c across epithelial, mesenchymal and intermediate cell lines in the NCI-60 panel (145) favors our proposed association of a hybrid E/M phenotype with (medium miR-200, medium ZEB) levels and places ZEB1 at the core of EMT decision-making. However, more quantitative experimentation, including due attention to cell-to-cell variability, is essential to validate certain predictions of the model.

The most fundamental prediction of the model – that of underlying tristability in the core EMT network – is bolstered by recent experiments showing that cell lines belonging to multiple cancer types can contain several subpopulations representative of E, M and hybrid E/M phenotypes (25, 43, 44), and has been echoed by other theoretical approaches to model EMT (151), despite some differences in modeling approaches and consequently other predictions of the model. It should be noted that our results still allow for a range of parameters (such as the \{E, E/M, M\}) phase where a hybrid E/M can be potentially ‘metastable’ as compared to E or M.

Elucidating the total number of intermediate EMT phenotype(s), their relative stability, and their corresponding expression levels of key EMT/MET players is an area
of active investigation. Further quantitative experimentation shall significantly contribute to resolving this granularity (8) by providing a fertile ground to integrate the ‘top-down’ models (those focusing on a few ‘core’ components of the network and predicting their emergent dynamics) (151–154) and the ‘bottom-up’ (those focusing on a modeling a much larger network and laying out all possible steady states of the network) (155–157) models of EMT regulatory network with experimental observations.

Chapter 3 takes the next step in characterizing the stability of the hybrid E/M phenotype and identifies a set of ‘phenotypic stability factors’ for a hybrid E/M phenotype via mathematical modeling. The predicted role of these factors in maintaining a stable hybrid E/M phenotype is then experimentally validated in H1975 lung cancer cells.

Note: Chapter 2 is largely based on the following publications (* denotes equal contribution):

Chapter 3

Stability of the hybrid E/M phenotype

3.1 Introduction: Novel modulators of EMT in developmental contexts

Recent evidence in cancer cell lines, circulating tumor cells (CTCs), and patient samples have indicated that a subpopulation of cells can co-express epithelial and mesenchymal markers (18, 19, 25, 38, 39, 42–44, 48, 49, 58–62, 75, 158, 159) therefore bolstering support for the existence of intermediate state(s) during EMT/MET. A predominance of this subpopulation in aggressive cancers such as melanoma and triple negative breast cancer (TNBC) and their correlation with enhanced invasive potential across cancer types suggest a hybrid E/M phenotype to be a hallmark of poor prognosis (9, 19, 44). Simultaneously, the role of a hybrid E/M phenotype in maintaining collective cell migration has gained further attention in EMT in the developmental (32–37) and tissue repair (30, 31) contexts, particularly in branching morphogenesis (33, 35).
Despite its crucial significance in both physiological and pathological EMT contexts, the hybrid E/M phenotype has not been comprehensively characterized. It has been proposed to be a ‘metastable’ or transient phenotype (95, 160), but recent experiments in mammary morphogenesis demonstrate that cells in terminal end buds (TEBs) that migrate collectively and form finger-like projections can maintain a hybrid E/M by the transcription factor(s) OVOL1/2 that operate(s) as a ‘critical molecular brake on EMT’ by preventing the ‘TEB cells that have gained partial plasticity’ from undergoing a full EMT. Knockdown of OVOL disrupts collective cell migration and promotes individual migration, reflective of a full EMT (35). On the other hand, overexpression of OVOL1/2 in prostate and breast cancer cells can drive an MET (106). Similarly, GRHL2, another well-known player in morphogenesis, can maintain collective migration at its endogenous levels, allows for an EMT when knocked down in cancer cells, and can drive an MET when overexpressed (110, 161).

Put together, these studies propose that OVOL1/2 and GRHL2 might behave as stabilizers for a hybrid E/M phenotype, or in other words, as ‘phenotypic stability factors’ (PSFs) (162). Therefore, here, we incorporated OVOL and GRHL2 in our theoretical framework to elucidate EMT dynamics. We also investigated the coupling of miR-145/OCT4 (microRNA-145/octamer binding transcription factor 4) loop with miR-200/ZEB because miR-145 can induce a partial EMT in prostate cancer cells and can drive an MET when overexpressed (163).
3.2 Theoretical framework to incorporate GRHL2 and OVOL1/2

We mathematically model the experimentally known regulations between miR-200/ ZEB and OVOL1/2, GRHL2, and miR-145/OCT4. The details of regulatory circuit construction, parameter estimation, and parameter sensitivity analysis (Figure S2.1, S2.2) are given in Appendix 2.

3.3 Results

3.3.1 H1975 lung cancer cells display a stable hybrid E/M phenotype

Motivated by many lung adenocarcinoma cell lines being categorized as intermediate (42) depending on ensemble measurements, we characterized three cell lines – H1299, H2291, and H1975 for their EMT status by staining them for canonical markers such as E-cadherin (CDH1) and Vimentin (VIM). H1299, characterized as a mesenchymal cell line, did not stain for E-cadherin but only for Vimentin, but H1975 and H2291 both stained for both CDH1 and VIM (Figure 3.1A). H2291 contained two subpopulations – one staining only for CDH1 (epithelial phenotype), and the others only staining for VIM (mesenchymal phenotype) (Figure S2.3), whereas H1975 cells co-stained for epithelial and VIM and CDH1, indicating a hybrid E/M phenotype at a single cell-level. More importantly, H1975 cells displayed this hybrid E/M phenotype stably for over two months in culture (Figure S2.4).

Since a hybrid E/M or partial EMT phenotype has been implicated in collective cell migration during both embryonic development and wound healing, we conducted a
scratch assay for H1975 and H1299 cell lines. H1299 cells moved largely as single cells, but H1975 cells formed finger-like projections and moved collectively (Figure 3.1B), hence strengthening the proposition that collective migration can be a prominent feature of cancer cells too, as also reflected by clusters of CTCs formed before entering the circulation (76, 79), and tumor budding (75, 85).

Figure 3.1: Characterizing the partial EMT phenotype. A. Expression of CDH1 (E-cadherin) and VIM (vimentin) examined by immunofluorescence staining. Scale bar 100 μm. B. Scratch assay of H1975 and H1299 depicting different cell motility
patterns. White arrows denote finger-like projections seen in H1975 cells. Panel i) shows the condition at the beginning of scratch assay (t = 0 hours) whereas ii) shows for t = 12 hours for H1299 and t = 16 hours for H1975 cells.

3.3.2 Mathematical modeling predicts GRHL2 and OVOL as ‘phenotypic stability factors’ (PSFs)

As a first step towards deciphering the effect of GRHL2 and OVOL on epithelial – hybrid- mesenchymal transitions, we incorporated them into our framework through the experimentally known links with the miR-200/ZEB loop, and evaluated the response of the coupled circuits to varying levels of SNAIL that acts as an input. The response of the coupled circuits to SNAIL is presented via a bifurcation diagram (Figure 3.2A,B), where lower levels (<100 molecules) of ZEB mRNA correspond to an epithelial phenotype, intermediate levels (~200-450 molecules) denote a hybrid E/M phenotype, and further higher values represent a mesenchymal phenotype. For low levels of SNAIL, cells attain only the epithelial (E) phenotype. With increasing values of SNAIL, cells first undergo a partial EMT to attain a hybrid E/M phenotype and then a complete EMT to attain a mesenchymal (M) phenotype. The range of SNAIL values for which the hybrid E/M phenotype exists is much larger for the (miR-200/ZEB/GRHL2) circuit as compared to that for the (miR-200/ ZEB) circuit (compare the green highlighted area in Figure 3.2B vs. that in Figure 3.2A). Most importantly, we now observe a range of SNAIL values for which the only possible state is hybrid E/M phenotype (region in the dotted rectangle), i.e. incorporating the effects of GRHL2 enables a range of parameter values (physiological conditions) under which most cells can attain a hybrid E/M phenotype. In other words, the model predicts that if the cells present in this parameter regime will be
sorted by FACS (Fluorescence Activated Cell Sorting), most of them will express both E and M markers, hence corresponding to a hybrid E/M phenotype. Similar results are observed for the (miR-200/ZEB/OVOL) circuit, and the (miR-200/ZEB/miR-145/OCT4) circuits (Figure 3.2C, S2.5, S2.6).

**Figure 3.2:** Dynamical system characteristics of miR-200/ZEB circuit when coupled with GRHL2 and miR-145/OCT4 separately. Bifurcation of mRNA levels of ZEB in response to SNAIL levels for A. miR-200/ZEB circuit, B. miR-200/ZEB/GRHL2 circuit, C. miR-200/ZEB/miR-145/OCT4 circuit, D. miR-200/ZEB/GRHL2 circuit when GRHL2 is inhibited by external signal SI, and E. miR-200/ZEB/GRHL2 circuit when GRHL2 is activated by external signal SA. Note the x-axis range change from (A) to (B), (C), (D), and (E). The region marked by green in panels (A), (B), (C) represents the range of SNAIL levels for which the hybrid E/M phenotype can exist alone or as one of the multiple possible phenotypes; and that marked by dotted rectangle in (B), (C), and (D) represents the range of SNAIL levels for which the hybrid E/M phenotype can exist alone. Corresponding cartoons have been drawn alongside the phenotype. Blue solid lines
denote stable steady states or phenotypes, and red dotted lines indicate unstable steady states. Yellow arrows in (D) and (E) indicate the SNAIL values at which cells can exit an epithelial phenotype for the control case (i.e. GRHL2 is not activated or repressed), whereas green arrows indicate the same for the case when GRHL2 is inhibited ((D)) or activated (E)).

Next, we investigated the effect of overexpressing or inhibiting these ‘phenotypic stability factors’ (PSFs) by considering an external activation (SA) or inhibition (SI) signal on GRHL2. Knockdown of GRHL2 destabilizes the hybrid E/M phenotype (Figure S2.7; note the lack of the region bound by dotted rectangle in the case of SI≠0 in Figure 3.2D) and can lead to a complete EMT, because the transitions to a complete EMT phenotype can happen at much lower levels of SNAIL in the absence of GRHL2. Conversely, overexpression of GRHL2 can induce an MET (Figure S2.7; note the much higher levels of SNAIL required to induce even a partial EMT when SA≠0 in Figure 3.2E). These predictions are consistent with experimental observations that a knockdown of OVOL and GRHL2 disrupts collective cell migration during the development of mammary gland and lung respectively (35, 161), and that an overexpression of GRHL2 and OVOL can drive MET in MDA-MB-231 (breast cancer) and PC3 (prostate cancer) cells respectively (106, 110).

3.3.3 Knockdown of GRHL2 or OVOL2 can induce a complete EMT in H1975 cells

To directly test our predictions in the stably hybrid E/M H1975 cells, we investigated the effect of silencing GRHL2 or OVOL2 in these cells. Treatment of H1975 cells with siRNA against GRHL2 or OVOL2 disrupts collective cell migration and forces
the cells to move more individually, suggesting a completion of EMT. Consistently, cells treated with siRNAs against either PSF lost the expression of CDH1 on both transcriptional and translational levels and gained ZEB1. The observed effect was more prominent in cells treated with siGRHL2, potentially because of the key role GRHL2 plays in lung development (161) (Figure 3.3A-C). Knockdown of GRHL2 or OVOL2 also inhibited cell proliferation (Figure S2.8) - a feature often associated with an EMT phenotype (64, 164, 165) - further indicating that ‘brake’ on full EMT is removed.

3.3.4 GRHL2 and OVOL2 may predict poor clinical outcome

Next, we examined the levels of GRHL2 and OVOL2 in PC3-Epi (epithelial clonal population) and PC3-EMT(mesenchymal clonal population) (Figure 3.3D, S2.9) – the two stable clonal cell lines derived from PC3 (106, 166), and observed significant changes in the ratio of PC3-Epi/PC3-EMT levels for GRHL2 (142.12x), OVOL1 (174.43x), OVOL2 (25.89x), ZEB1 (0.02x) and E-cadherin (50.43x), indicating that EMT/MET is linked with differential expression of all these players. These results are reminiscent of the observation that GRHL2 and OVOL1/2 are highly correlated with the ‘NCI-60 consensus epithelial’ (NEC) signature (167) – a list of genes such as CDH1 (E-cadherin) that are important in maintain adherens and/or tight junctions between epithelial cells. Furthermore, we investigated the NCI-60 cohort that has been categorized into groups of epithelial, hybrid E/M and mesenchymal cell lines based on their CDH1/VIM ratio(118). The levels of ZEB1, GRHL2, OVOL2 and CDH3 (P-cadherin, a proposed marker of hybrid E/M phenotype(168) that has 16.02x levels in PC3-Epi as compared to PC3-EMT) are all different in a statistically significant manner across the
three different categories (Figure S2.9). Further, the levels of GRHL2, OVOL2, and CDH3 all positively correlate with one another and all of them negatively correlate with ZEB1 (Figure 3.3D). The strongest association was observed between GRHL2 and OVOL2, further consolidating our predictions about their similar roles in shepherding epithelial-hybrid-mesenchymal transitions. Besides, in a panel of 877 cell lines (106) from the Cancer Cell Line Encyclopedia (CCLE) (169), OVOL1/2 is highly correlated with GRHL2 and CDH3 (positively) and ZEB1 (negatively). Also, GRHL2, OVOL2, ZEB1, and many members of the miR-200 family are enlisted as top 25 regulators of E-cadherin expression (170).

Finally, we asked whether levels of GRHL2, OVOL1/2, and CDH3 either alone or in combination may predict patient outcome across multiple datasets. Indeed, high levels of the PSFs GRHL2 and OVOL2, as well as those of the proposed marker for a hybrid E/M phenotype CDH3, can correlate with poor overall survival (OS), metastasis-free survival (MFS), and relapse-free survival (RFS). Their low levels, indicative of a completely mesenchymal phenotype as elucidated above, associate with a better survival, thereby arguing that a complete EMT might not be as strongly associated with aggressiveness as a hybrid E/M phenotype (Figure 3.4, S2.10). Also, these survival curves resonate with the observations that higher levels of GRHL2 correlate with shorter RFS in breast cancer patients (171), with lower OS and RFS in colorectal cancer patients (172), and that a GRHL2-mediated gene set pair (173) can “effectively stratify patients showing significant differences in metastasis-free survival” in a set of 1678 independent human breast cancer samples.
Figure 3.3: Knockdown of GRHL2 and OVOL2 in H1975 cells, and expression values of GRHL2, CDH3, and OVOL in different PC-3 clones. A. Scratch assay of H1975 cells for the control case, and when treated with siRNA against GRHL2 or OVOL2, depicting different cell motility patterns. B. Expression of CDH1 (E-cadherin, red) and VIM (Vimentin, green) examined by immuno-fluorescence staining. Scale bar 100 μm. The blue color stains DAPI. C. Quantitative RT-PCR for CDH1, VIM, GRHL2, and OVOL2 after and before treatment with siRNAs against GRHL2 or OVOL2. Top panel is for siRNA against GRHL2, bottom one for that against OVOL2. H1975si shows the case when cells are treated with siRNA, H1975 NC denotes the negative control, and H1975m denotes mock case. *, p ≤ 0.05; **, p < 0.005; and ***, p < 0.0005. Error bars represent standard deviation, n = 3. D. (top) Fold-change in expression levels (log2) of GRHL2, OVOL2, CDH3, and ZEB1 in PC3-Epi vs. PC3-EMT, and PC3-EMT-OVOL2 vs. PC-EMT clonal cell lines. (bottom) Correlation analysis for GRHL2, OVOL2, CDH3, and ZEB1 expression in NCI-60. Pearson correlation coefficients (r) and p-values (two-tailed) are given.
Figure 3.4: Survival analysis. Overall survival and metastasis-free survival for the expression of GRHL2, OVOL2 and CDH3 individually in multiple tissue types - A., B. GSE13507 (n = 164), C. GSE7390 (n = 197), D., E. TCGA_KIRC (n = 505), F.TCGA_COAD (n = 120).

The correlation of ‘phenotypic stability factors’ and consequently a stable hybrid E/M phenotype with poor patient outcome is also reminiscent of observations made for transmembrane glycoprotein - podocalyxin (PODXL). PODXL overexpression – an independent prognostic factor in multiple tumor types (174–176) – can stimulate collective migration in a 2-D monolayer culture, and a collective budding and invasion phenotype in 3-D culture (177). It enhances the invasiveness of breast and prostate cancer cell lines (178) and is enriched at the leading edge of migrating cells (179). Furthermore, its role in regulating the frequency of tumorsphere-initiating cells (180) also mimics the proposed role of GRHL2 and OVOL2 to maintain the ‘stemness window’ around mid-point of the ‘EMT axis’, i.e. a hybrid E/M phenotype (see Chapter 4).
3.3.5 Proposing network motifs to find more ‘phenotypic stability factors’

Intrigued by the similar role being played by GRHL2, OVOL and miR-145 as PSFs for the hybrid E/M phenotype, we analyzed the topologies of the (miR-200/ZEB/GRHL2), (miR-200/ZEB/OVOL) and (miR-200/ZEB/miR-145/OCT4) circuits for similarities and differences. For an easy comparison, (miR-200/ZEB/miR-145/OCT4) circuit was effectively reduced to (miR-200/ZEB/miR-145) circuit such that similar to GRHL2 and OVOL2, miR-145 inhibits ZEB and is inhibited by ZEB in a relatively weak manner as compared to the inhibition of miR-200 by ZEB (Figure S2.11).

These circuits have two key differences – (a) GRHL2 has no self-regulation, OVOL self-inhibition, and miR-145 self-activates (considering the effective circuit), and (b) GRHL2 does not inhibit miR-200, but OVOL and miR-145 (in the effective circuit) do. Thus, we investigated how the two hypothetical links – self-regulation of GRHL2 and inhibition of miR-200 by GRHL2 – affect the range of SNAIL levels for which the hybrid E/M phenotype would exist.

Having included the hypothetical self-regulation of GRHL2, we observe that the total area corresponding to the monostable {E/M} phase and multi-stable phases with hybrid E/M as one of the phenotypes increases with a decrease in strength of self-inhibition and/or an increase in that of self-activation (area bound by black dots in Figure 3.5A, left panel). On the other hand, including the hypothetical inhibition of miR-200 by GRHL2 does not largely affect the total area corresponding to phases with hybrid E/M as one of the phenotypes (area bound by black dots in Figure 3.6A, middle panel). Therefore, neither of the two links – inhibition of miR-200 by GRHL2 and self-regulation
of GRHL2 – qualitatively changes the behavior of the PSFs. Based on these results, we propose two network motifs that can be used to identify PSFs, i.e. if a particular TF or miR couples with (miR-200/ZEB) in either of these topologies, it is highly likely to behave as a PSF (Figure 3.5B, i-ii). In these motifs, a PSF forms a double negative feedback loop with ZEB, may inhibit miR-200, and may self-regulate (either activate or inhibit, as shown by the dot at the end of the self-regulatory link).

Figure 3.5: Network motifs that help maintain the hybrid E/M phenotype. A. Top row denotes the different circuits that are investigated; the link whose strength is varied is depicted in red. Bottom row shows the phase-diagram of the miR-200/ZEB/GRHL2 circuit when driven by SNAIL and including a hypothetical self-regulatory link for GRHL ($\lambda_{G.m_i}$ denotes the strength of the self-regulation; $\lambda_{G.m_i} > 1$)
indicates self-activation, $\lambda_{G,m_0} < 1$ implies self-inhibition, and $\lambda_{G,m_0} = 1$ indicates no self-regulation) (left), phase diagram of the miR-200/ZEB/GRHL2 circuit when driven by SNAIL and a hypothetical inhibitory link from GRHL2 to miR-200 (the smaller the value of $\lambda$, the stronger the inhibition) (center), and phase diagram for the miR-200/ZEB/OVOL2 circuit when driven by SNAIL and including a hypothetical self-regulatory link for OVOL ($\lambda_{G,\mu_{200}}$ indicates the strength of self-regulation: $\lambda_{G,\mu_{200}} < 1$ implies self-inhibition, $\lambda_{G,\mu_{200}} > 1$ implies self-activation, and $\lambda_{G,\mu_{200}} = 1$ shows no self-regulation) (right). In all these phase diagrams, the black dots bound the area of all phases that contain E/M as one of its phenotypes, and dark green region shows the phase when hybrid E/M phenotype need not co-exist with other phenotypes - {E/M} B. Proposed network motifs for identifying PSFs. The dot at the end of the self-regulatory link indicates that the PSF can potentially either self-activate or self-inhibit.

In addition to these proposed PSFs, we propose another network motif for identifying PSFs – a player that can inhibit both miR-200 and ZEB (Figure 3.5B, iii). This motif is proposed based on the analysis of miR-200/ZEB/OVOL circuit with the inhibition of OVOL by ZEB is missing; ZEB inhibiting OVOL has not been reported, for example, in mammary morphogenesis and epidermal development (35, 181). In this proposed motif, OVOL can either self-inhibit or self-activate; relieving the self-inhibition and/or increasing the self-activation only leads to a larger area corresponding to the phases that contain E/M as one or the only phenotypes (area bound by black dots in Figure 3.5A, right panel). We further validated the specificity of the network motifs by analyzing other topologies, for instance, if a molecular player forms mutually inhibitory loop with miR-200 instead of ZEB, or activates both miR-200 and ZEB instead of inhibiting them, the hybrid E/M phenotype is not stabilized (Figure S2.11-2.13). Put together, these three proposed network motifs can be used to identify additional factors that can stabilize a hybrid E/M phenotype.
3.4 Discussion

Here, using an integrated computational modeling and experimental approach, we present OVOL2 and GRHL2 as examples of ‘phenotypic stability factors’ (PSFs) for a hybrid E/M phenotype that can act as “critical molecular brake on [a complete] EMT”, and suggest that a partial EMT or hybrid E/M phenotype need not always be a ‘metastable’ (95, 160) state en route EMT. The number, relative stability, and expression profile of such hybrid or intermediate state(s) still remain open questions (151–156), but the arrangement of two PSFs OVOL and GRHL2 in a coherent feed-forward loop (FFL) topology(182) (GRHL2 can activate OVOL during nephric bud development and both of them can induce CDH1) – a common network motif that acts as a “stabilizer of target gene expression” (183) further strengthens the emerging notion that a hybrid E/M phenotype can “define [a] normal cell identity” and is not “necessarily an unstable transitory state” (184).

The identification of PSFs presented here is in no way comprehensive. Rather, we present GRHL2, OVOL2 and miR-145 as examples of PSFs that can then be generalized to suggest a set of network motifs that can be used to search for other PSFs. Recent experiments propose another possible PSF – ∆Np63α – that can activate both SNAI2 and inhibit ZEB1 by activating miR-205, thus inducing a partial rather than a complete EMT in breast cancer cells (184). Importantly, P-cadherin (CDH3), a proposed marker for hybrid E/M phenotype, is regulated by ∆Np63α (168). Activation of p63 gene by GRHL2 in keratinocytes (185), and co-expression of ∆Np63α, Slug, and P-cadherin in the myoepithelial cells (168, 184) further strengthens the hypothesis that ∆Np63α can act
as a PSF. Although some of the proposed PSFs can upregulate others in different contexts (for instance, GRHL2 can activate OVOL2 and p63), not all of them are necessarily present or active in the same biological context. Hence, whether two or more PSFs function redundantly or synergistically is highly likely to be a tissue-specific trait. Furthermore, a proposed PSF does not necessarily stabilize hybrid E/M phenotype, if it couples to (miR-200/ZEB) in a topology quite different from the network motifs proposed (107, 111).

Our results also suggest caution in labelling a cell line or group of cells ‘hybrid E/M’. H1975 and H2291 both were classified as hybrid based on population-based measurements (42), but one of them is an admixture of E and M cells, whereas the other predominantly contains hybrid E/M cells. These two possibilities – mixtures of E and M vs. hybrid E/M as single-cell level – are not mutually exclusive; recent experiments indicate a co-existence of all three phenotypes in prostate, breast and lung cancer cell populations (25, 43, 44). Most importantly, H1975 cells can maintain their hybrid E/M phenotype for over two months in culture in the presence of PSFs and display collective migration, indicating their stability. Similar co-expressing cells have been reported in multiple cell lines (43, 44, 152), but most previous reports on partial EMT (38, 42) have been at an ensemble level, therefore being inconclusive whether they contain a subpopulation of truly hybrid E/M cells.

Overall, using a quantitative systems biology approach, we present three ‘phenotypic stability factors’ (PSFs) – OVOL2, GRHL2 and miR-145 – that can help maintain a hybrid E/M phenotype and can potentially increase the likelihood of hybrid E/M
phenotype to associate with higher tumor-initiation potential (work not discussed in detail in this thesis) (99, 100, 186). This proposed dual role of the PSFs can both facilitate collective cell migration of clusters of CTCs and enrich their tumor-initiation potential, thus significantly increasing the metastatic load which is supported by the clinical observations that patients with relatively high levels of one or more of GRHL2, OVOL2, and CDH3 have lower overall survival, metastasis-free survival, and progression-free survival. Therefore, these PSFs can be valuable targets to break the CTC clusters – the real ‘bad actors’ of metastasis that possess multiple advantages during metastatic dissemination – resistance to anoikis, enhanced tumor-initiating potential, ability to carry immune cells along to evade an immune attack, and finally the ‘priming’ for subsequent rounds of metastasis (9). From a clinical standpoint, these results suggest a rethinking in diagnostic strategy. With advancements such as the FDA approval of the first ‘liquid biopsy’, isolating and characterizing the clusters of Circulating Tumor Cells (CTCs) may be a more effective and much-needed approach than counting total CTCs to monitor disease aggressiveness and treatment effectiveness and eventually stratify the metastatic risk (76, 77, 87, 187).

The identification of these PSFs further draws attention to the emerging paradigm that EMT is not an ‘all-or-none’ process and can help contribute to resolving conflicting observations about EMT and tumor-initiation potential (4, 44, 89–94). The first attempt, to the best of our knowledge, to elucidate the underlying connections between EMT and tumor-initiation potential via an integrative predictive modeling framework is presented in Chapter 4.
Note: Chapter 3 is largely based on the following publications (* denotes equal contribution):


The *in vitro* experiments presented in this chapter (Fig 3.1, 3.3) were conducted by Satyendra C Tripathi and Muge Celiktas from Dr. Hanash’s group at the University of Texas MD Anderson Cancer Center, Houston, TX.
Chapter 4

Enhanced tumor-initiation potential of the hybrid E/M phenotype

4.1 Introduction: Coupling EMT and stemness regulatory networks

Metastasis and tumor relapse remain the clinically insuperable aspects of cancer treatment. While aberrant activation of EMT and MET has been shown to fuel metastasis, tumor relapse is believed to be caused by the exit from dormancy of the quiescent therapy-resistance metastasis-seeding or tumor-initiating cells, also referred to as ‘Cancer Stem Cells’ (CSCs) (188). These cells that possess ‘stemness’, i.e. properties similar to stem cells, may stay dormant for long periods of time, can self-renew, and possess tumor-initiation and long-term clonal repopulation ability (189). However, especially in the context of cancer, ‘stemness’ is not a fixed trait of some privileged cells, rather CSCs and non-CSCs can dynamically interconvert into one another and drive tumor invasion (190–193). Thus, CSCs can give rise to significant heritable phenotypic variation (194).
A mechanism through which cells have been proposed to gain ‘stemness’ is a complete EMT. The EMT-stemness coupling was first observed in immortalized human mammary epithelial cells; similar observations were made in carcinomas such as pancreatic and hepatocellular cancer later (4, 89, 90). However, the fundamental question whether hybrid E/M cells can also attain stemness remains open.

Addressing this question requires unraveling the operating principles of the coupling between EMT and stemness. As detailed in Chapter 2, EMT is regulated by the mutually inhibitory feedback loop miR-200/ZEB that enables three phenotypes – epithelial, mesenchymal and hybrid E/M. Similarly, stemness is regulated by a mutually inhibitory feedback loop between the RNA binding factor LIN28 and microRNA let-7(195). The LIN28/let-7 and miR-200/ZEB circuits are connected via multiple links – miR-200 inhibits LIN28 (196), and LIN28 activates the pluripotency factor OCT4 (197) that can upregulate miR-200 (198) (Figure 4.1). It has been pointed out consistently in both embryonic development and cellular reprogramming studies that cells with intermediate levels of OCT4 are closely associated with maximum stemness or pluripotency; both very high and very low levels of OCT4 drive the cell to differentiation (199–202). Therefore, we consider a range of intermediate levels of OCT4 as our ‘stemness window’, and focus our attention on the coupling of EMT and stemness networks. This coupling between EMT and stemness can be modulated by oncogene c-MYC and inflammatory response molecule NF-kB that can alter the dynamics of LIN28/let-7 feedback loop by affecting the production rates of LIN28 and/or let-7 (203–206).
**Figure 4.1: The regulatory network coupling EMT and stemness.** (a) Novel modes of regulation in the LIN28/let-7 circuit. LIN28 promotes its own translation (green solid line), and inhibits let-7 processing (orange solid line); let-7 promotes its own processing (red solid line) and inhibits LIN28 translation (black dashed line). NF-κB activates both LIN28 and let-7, whereas c-MYC activates LIN28 and inhibits let-7. miR-200 inhibits LIN28, that translationally activates OCT4 (orange solid arrow) that activates miR-200. (b) Effective circuit showing the LIN28/let-7 double negative feedback loop that receives external input signals from miR-200, c-MYC and NF-κB; and has OCT4 as the output. The activation of miR-200 by OCT4 has not been considered in our current framework. A blue solid line shows conversion of one species to another, a solid black arrow shows transcriptional activation, a solid black bar denotes transcriptional repression, and a black dashed line shows microRNA-mediated translational repression.

### 4.2 Theoretical framework to study EMT-stemness interplay

LIN28 is a RNA binding factor that can promote its own translation and inhibit the processing of let-7, whereas let-7 can promote its own processing (details given in Appendix 3). Therefore, LIN28 and let-7 are both self-activating elements with novel modes of regulation. Motivated by the need to capture these novel regulatory modes of the LIN28/let-7 feedback loop, we developed a theoretical framework (details given in Appendix 3) starting from our framework for miRNA-mediated translational repression(97). With this framework, we first investigate the dynamics of stand-alone
LIN28/let-7 circuit as driven only by miR-200, and subsequently the dynamics when the circuit is driven by miR-200 in combination with c-MYC or NF-kB. It should be noted that we do not include the regulation of miR-200 by OCT4 in the current model. Parameter estimation and sensitivity analysis is given in Appendix 3(Figure S3.1-S3.4).

4.3 Results

4.3.1 LIN28/let-7 behaves as a three-way switch

As the first step, we analyzed the LIN28/let-7 circuit when driven by a constant level of miR-200. A typical phase space diagram (Figure 4.2A) illustrates the coexistence of three stable states – (i) (high LIN28, low let-7) – referred to as (1, 0) or up (U) state, (ii) (low LIN28, high let-7) – referred to as down (D) or (0, 1) state, and (iii) (medium LIN28, medium let-7) – referred to as down/up (D/U) or (½, ½) state. These results about LIN28/let-7 behaving as a three-way switch are reminiscent of experiments showing that cells can concomitantly express both LIN28 and let-7 – (medium LIN28, medium let-7) – in addition to expressing one or the other exclusively (207, 208).

Because miR-200 directly targets LIN28, we expected some overlap between the three states of EMT core circuit – (E, M, and E/M) with those of the LIN28/let-7 circuit (D, U, and D/U). To test our expectation, we varied the levels of miR-200, an external signal to (LIN28/let-7) circuit. As expected, at very high levels of miR-200, corresponding to an epithelial phenotype, only the LIN28/let-7 circuit exhibits only one state D – (0, 1). Similarly, at very low levels of miR-200 corresponding to a mesenchymal phenotype, only the U – (1, 0) exists. However, at intermediate levels of
miR-200 corresponding to a hybrid E/M phenotype, the D/U – (½, ½) state can co-exist along with D and U states. Also, some range of miR-200 values enable the coexistence of only D and U states (Figure 4.2B). The bifurcation diagram shown here also reminds us of the asymmetry in state switching as observed between EMT (decrease in miR-200) and MET (increase in miR-200).

The proposed association of the (high LIN28, low let-7) with a mesenchymal phenotype and that of the (low LIN28, high let-7) with an epithelial phenotype is consistent with experimental observations that epithelial cells have upregulated let-7 but low LIN28, while mesenchymal cells have upregulated LIN28 but low let-7(196, 209). Therefore, we propose that the hybrid E/M cells are likely to be in the (medium LIN28, medium let-7) or D/U state.

Figure 4.2: Dynamical behavior of the LIN28/let-7 circuit. (a) Nullclines and three possible steady states as predicted by the model for fixed miR-200 = 5000 molecules. Red nullcline is for dB/dt = 0, and black nullcline is for dμ/dt = 0 (see the equations in Appendix 3). Green solid dots denote possible states (stable fixed points), and green hollow circles denote unstable fixed points. The states are U—(1, 0) or (high LIN28, low let-7); D—(0, 1) or (low LIN28, high let-7); and D/U—(1/2, 1/2) or (medium
LIN28, medium let-7). (b) Bifurcation of LIN28 protein levels in response to inhibition by miR-200. It illustrates the possible coexistence of three states between epithelial (high miR-200 levels) and mesenchymal (low miR-200 levels). Note that for the parameters used in this figure (c-MYC = 0 and NF-κB = 0), D/U state exists for miR-200 levels from 3000 to 7000 molecules, levels that are consistent with typical levels of miR-200 corresponding to the E/M phenotype (5000–15,000 molecules (97)). As is shown later, in the presence of NF-κB, the range of existence of the D/U state can increase from 4000 to 12,000 miR-200 molecules. Starting from the E phenotype, at bottom right part of the blue curve, and decreasing miR-200 levels, the circuit undergoes a transition from the D—(0, 1) state into the D/U—(1/2, 1/2) state. Further decrease in miR-200 to levels that correspond to M phenotype leads to a transition from the D/U state into the U state. Starting from U state and increasing miR-200 levels, the circuit directly undergoes a transition into the D state at miR-200 level that corresponds to the E phenotype.

4.3.2 Effect of c-MYC on coupling between LIN28/let-7 and miR-200/ZEB

Figure 4.3: Circuit response to external signals c-MYC and miR-200. (a) Bifurcation diagram showing the dependence of the level of LIN28 on c-MYC when LIN28 is inhibited by constant miR-200 level = 7000 molecules. Blue solid lines indicate stable states, and red dotted lines denote unstable states. Corresponding EMT phenotypes have been indicated for every stable state. (b) Required levels of miR-200 for the D/U—(1/2, 1/2) state to exist for different levels of c-MYC. Red dots show minimum miR-200 levels for which the D/U state can exist, and blue dots are for corresponding maximum values. The area within the green horizontal dashed lines represents the range of miR-200 levels that are associated with the hybrid E/M phenotype (5000–15,000 molecules) (96). Corresponding phenotypes have been indicated for different states (E, M, E/M).
depending on miR-200 levels. Brown vertical dotted line denotes the maximum estimated levels of c-MYC in a cell (approx. 30,000 molecules (209)).

Figure 4.3 shows the effect of c-MYC at different levels of miR-200. At miR-200 = 7000 molecules (a typical level for the E/M phenotype (96)), the D/U—(1/2, 1/2) state exists only for a small range of c-MYC; higher c-MYC levels drive the system to the U state exists and the circuit is monostable (Figure 4.3A). Therefore, for only c-MYC levels less than typical levels (approx. 30,000 molecules (209)), the miR-200 levels for which D/U state exists match with those corresponding to a hybrid E/M phenotype (blue region highlighted in Figure 4.3B). For c-MYC levels higher than those typical values, the levels of miR-200 required to maintain a D/U state are much higher and correspond to epithelial phenotype. Thus, at high c-MYC levels, D/U state corresponds with an epithelial phenotype. Further, when miR-200 levels are too high or too low, the range of existence of D/U state decreases significantly and the circuit becomes bistable or monostable (Figure S3.5).

4.3.3 Effect of NF-kB on coupling between LIN28/let-7 and miR-200/ZEB

Next, we investigated the effect of NF-kB on the LIN28/let-7 standalone circuit and then on the EMT-stemness coupling. For intermediate levels of miR-200, the (D/U) or (½, ½) state exists for a significant range of values of NF-kB (Figure 4.4A). Similarly, for intermediate levels of NF-kB, the (D/U) state exists for a significant range of values of miR-200 (4000-12000 molecules) (Figure 4.4B). With increasing levels of NF-kB, the range of miR-200 levels for which the D/U state exists increases (Figure 4.4C). More
importantly, this range of miR-200 levels for the existence of D/U state very well overlaps with the range of miR-200 levels corresponding to a hybrid E/M phenotype (97), therefore suggesting two effects of NF-kB: (a) cells maintaining a D/U state, and (b) higher correspondence between the D/U state and the hybrid E/M phenotype.

Further, we investigated how varying the levels of miR-200 affect the range of NF-kB values for the existence of D/U state. At higher levels of miR-200, the D/U state exists for an increasing range of levels of NF-kB. However, at very high levels of miR-200 commensurate with an epithelial phenotype (>15000 molecules (97)), the minimum amount of NF-kB molecules required to enable the existence of the D/U state tend to reach maximum levels of NF-kB as experimentally observed (35,000 molecules in non-cancer cells and potentially up to 50,000 molecules in cancer cells (210)) (Figure 4.4D).

To further unravel how the likelihood of gaining stemness depends on NF-kB and miR-200, we plotted a phase-space diagram (two-parameter bifurcation diagram) (Figure 4.5A). It contains the following phases – {D}, {U}, and {D/U} (only one state exists), {D, U} and {D/U, U} (co-existence of two states) and {D, U, D/U} (co-existence of all three states). The phases containing the D/U state – both {D, U, D/U} and {D/U, U} – exist for intermediate values of miR-200 and the range of miR-200 values for their existence increases with increasing values of NF-kB, reiterating that increasing values of NF-kB stabilize D/U state and promotes the overlap of D/U with hybrid E/M phenotype.
Figure 4.4: Circuit response to external signals NF-κB and miR-200. (a) Bifurcation diagram shows the dependence of the level of LIN28 on NF-κB when LIN28 is inhibited by constant miR-200 level = 6000 molecules. (b) Complementary bifurcation diagram showing the dependence of the level of LIN28 on miR-200 when the circuit is driven by constant level of NF-κB = 25,000 molecules. Note that the required levels of miR-200 for the existence of D/U state (4000–12,000 molecules) overlap well with the levels of miR-200 for the existence of E/M phenotype (5000–15,000 molecules). Blue solid lines indicate stable states, and red dotted lines denote unstable states. Corresponding phenotypes (E, M, E/M) have been indicated for every stable state. (c) Range of miR-200 levels for which the D/U—(1/2, 1/2) state exists, for varying values of NF-κB. Red dots show minimum values of miR-200 for which this state exists, and blue dots for corresponding maximum values. Corresponding phenotypes have been indicated for different states (E, M, E/M) depending on miR-200 levels. (d) Range of NF-κB values for which D/U state exists for different values of miR-200. Red dots are for minimum values of NF-κB for which this state exists, and blue dots are for corresponding maximum values. Arrows denote that the corresponding maximum levels for NF-κB are greater than 60 000 molecules. Brown horizontal dotted line denotes the maximum estimated levels of NF-κB in a cell (50,000 molecules (210)). Areas within the green dashed lines—horizontal in (c) and vertical in (d)—represent miR-200 levels that are associated with hybrid E/M phenotype.

4.3.4  ‘Stemness window’: Hybrid E/M can be more stem-like than a fully M state

Finally, we computed the levels of stemness marker OCT4 as a function of LIN28, and chose the representative levels of relative OCT4 levels that correspond to a higher likelihood of stemness (0.25-0.65 relative to saturation levels of OCT4 when OCT4 is activated by maximal LIN28 values), and overlapped the regions of stemness on the phase diagram plotted for varying miR-200 and NF-κB (Figure 4.5B). Results for this ‘guessed range’ of OCT4 are shown here to illustrate the concept; the range of OCT4 levels defining the ‘stemness window’ can be cancer or even patient specific.
Figure 4.5: Cells in hybrid E/M state are highly likely to be stem-like. (a) Phase-space diagram of the response of LIN28/let-7 circuit to miR-200 and NF-κB. Each phase corresponds to a different combination of coexisting phenotypes and is denoted by a different colour. For example, in phase \{D\}, cells are in D state; and cells in phase \{D, U, D/U\} can adopt any of the three states—D, U or D/U. (b) Mapped regions for likelihood of stemness, as defined by a range of intermediate OCT4 levels (relative levels $= 0.25$–$0.65$). Cells in D/U state in the entire \{D, U, D/U\} phase and part of the \{D/U, U\} phase (as marked with black dotted lines, and bound by black solid lines in yellow region, and black solid lines at the boundary of pink and light green region) have high likelihood of stemness. In addition, cells in the U state are likely to be stem-like in a part of the \{D, U\} region (as marked with red dotted lines and bound by red solid lines in light green region, and red solid lines at the boundary of light green and blue region). Cells in both U and D/U states are both likely to be stem-like in the small area at the bottom (bounded with red and black lines) in \{D, U, D/U\} phase.

We find high stemness likelihood in the \{D, U\}, \{D/U, U\}, and \{D, U, D/U\} phases. In these multi-stable phases, we further investigated which state of the LIN28/let-7 corresponded to OCT4 levels lying in the ‘stemness window’, and identified the region associated with stemness when cells are in D/U state and that when cells are in a U state.
Notably, the area corresponding to stemness is much larger for the D/U state as compared to the U state, suggesting that cells in the D/U state can also gain stemness in addition to those in the M state. Moreover, with increasing values of NF-kB, the area associated with stemness decreases for the U state but that for the D/U state increases, thus strongly arguing that NF-kB can facilitate the association of the D/U state and/or the hybrid E/M phenotype with higher stemness (Figure 4.5B).

Therefore, the model predicts that at least for some cancer cases, cells in a hybrid E/M phenotype may be more likely to gain stemness as compared to those in a mesenchymal phenotype. To further validate this prediction using dynamical simulations, we analyzed the dynamics of LIN28 /let-7 circuit in response to decreasing levels of miR-200 thereby representing an EMT (Figure 4.6). Initially, the cells are in a D state, and as the levels of miR-200 decrease, they switch to attain a D/U state and maintain it for around 2 days, before transitioning to attaining a U state as miR-200 levels further decrease towards the levels that correspond to a mesenchymal phenotype (Figure 4.6A). A similar treatment of cells but with low levels of NF-kB shortens the duration for which they can maintain a D/U state (Figure 4.6C), thereby reiterating that NF-kB acts to stabilize a D/U state of cells. We also plotted the levels of OCT4 during EMT, and observed that some cells in a D/U state, but not necessarily those in U state, have OCT4 levels in the range of ‘stemness window’ (Figure 4.6B, D). Thus, at least for the ‘guessed range’ of OCT4 and following the dynamic trajectory of cells in these simulations, it is less likely that cells in a mesenchymal phenotype retain their ‘stemness’.
4.4 Discussion

Here, we present the first step towards computational modeling of the crucial inter-connections between EMT and stemness by developing a theoretical framework to study the characteristics of LIN28/let-7 feedback loop and its coupling with miR-200 and NF-kB. The model predicts that the LIN28/let-7 loop is tristable, and it states usually correspond to the three states of the miR-200/ZEB loop – (low LIN28, high let-7) with...
epithelial, (high LIN28, low let-7) with mesenchymal, and (medium miR-200, medium let-7). The proposed association is consistent with observations made for epithelial and mesenchymal phenotypes (196, 209), but a quantitative analysis of LIN28 and let-7 for cells in a hybrid E/M phenotype remains to be done.

Next, we predict that due to its role in both stabilizing the D/U state and increasing its association with the hybrid E/M phenotype, NF-kB might enhance the likelihood for cells to attain a hybrid E/M phenotype. This prediction is commensurate with the recent observation that the triple negative breast cancer (TNBC) that has constitutively higher expression of NF-kB (211) also has the highest percentage of hybrid E/M cells in tumor biopsies across all the breast cancer subtypes (18).

Most importantly, the model predicts that cells in a hybrid E/M phenotype or equivalently D/U state are highly likely to gain stemness, many times even more so than mesenchymal cells. Indeed, the co-expression of epithelial and mesenchymal markers on trophoblast stem cells, hepatic progenitor/stem cells, adult epithelial cells, and neoplastic stem cells agree with our prediction (212–215). Moreover, our prediction is also consistent with observations made for EMT-stemness interplay in non-cancer contexts, such as (a) during developmental (type I) EMT (20), renal glomerular epithelial cells undergo a partial EMT to attain the traits of bipotent progenitors (216), and (b) during EMT contributing to tissue repair (type II EMT (20)), hybrid E/M cells behave as bipotent adult hepatic progenitors during injury and repopulate the rat liver (217).

Finally, recent observations in tumor-initiation potential or stemness of cancer cells also resonate well with the proposition that the ‘stemness window’ lies somewhere
close to midway on the ‘EMT axis’ (Figure 4.7) (9, 88). First, Grosse-Wilde et al. (44) showed that cells co-expressing canonical epithelial and mesenchymal genes enhance mammosphere formation independent of the breast cancer subtype. Second, Ruscetti et al. (61) isolated hybrid E/M cells in vivo and showed that their tumor-initiation potential was comparable or even higher than that for completely mesenchymal cells. Later, the authors illustrated that upon culturing the three subpopulations (E, E/M and M) separately, while a majority of E and M cells maintain their initial state, more than 70% of hybrid E/M cells transition into either E or M within 24 hours, reflecting their augmented stemness or plasticity (25). Third, Strauss et al. illustrated that a subpopulation of cells in a hybrid E/M phenotype in primary ovarian cultures and tumors in situ can be multipotent, co-express markers of different lineages, and can drive aggressive tumor growth in vivo by self-renewing and generating more differentiated cells. Last but not least, although Liu et al. (218), Biddle et al. (158) and Maenz et al. (219) did not consider the possibility of existence of hybrid E/M cells, they indicated that the cells locked in a completely mesenchymal state have significantly compromised plasticity and/or tumor-initiation potential.

Figure 4.7: Association of partial EMT with stemness. (A) “EMT gradient” model proposed by Ombrato and Malanchi (88) where stemness is maintained within a
window between a fully differentiated epithelial cell and a fully de-differentiated mesenchymal cell [Figure adapted from Ref. (88), cartoons included]. (B) Stemness or tumor-initiating potential of E, E/M, and M phenotype, or variation of stemness during EMT progression (brown line).

The ‘stemness window’ positioned midway on the ‘EMT axis’ also potentially reconciles conflicting observations that EMT increases or decreases the tumor-initiation potential (4, 89–92), as most of these studies categorized EMT as a binary process, therefore unknowingly merging the hybrid E/M phenotype with epithelial and/or mesenchymal phenotypes. Further, the ‘stemness window’ positioning is expected to be a contributing factor underlying the observations that transient SNAIL (220) or TWIST (221) activation, but not necessarily a sustained activation, leads to aggressive behavior. Overall, the exact mapping between EMT and stemness seems to be context-dependent and complex (222), therefore, as presented in our expanded model of EMT-stemness coupling (not discussed in detail here) (186), the ‘stemness window’ is tunable and can move to either the epithelial or mesenchymal side of the ‘EMT axis’. This tuning of ‘stemness window’ can be mediated by different coupling strengths between the two tristable circuits (LIN28/let-7) and (miR-200/ZEB), various ‘phenotypic stability factors’ such as OVOL1/2 and GRHL2, and the effect of activation of OCT4 by miR-200. Such fine-tuning affords the cells plasticity in mediating epithelial-mesenchymal transitions as well as modulating their ‘stemness’. With EMT being no longer considered to be an ‘all-or-none’ process (24) and stemness being acknowledged as a reversible trait of cells rather than a fixed state (223), such multistability might enable cancer cells to adapt to their ever changing microenvironment. Nevertheless, the proposed association of hybrid
E/M with higher tumor-initiation potential is expected to underlie, at least in part, the reported 50-fold higher metastatic potential of the clusters of circulating tumor cells (CTCs)(76).

It should be highlighted the ability of cancer cells to form clusters depends on at least two factors – a hybrid E/M phenotype that couples their adhesion and migration, and the intercellular communication that can spatially coordinate the cluster formation. The intracellular networks underlying the former have been the focus of the thesis so far. Chapter 5 integrates these intra-cellular networks with those that mediate cell-cell communication such as the Notch pathway.

Note: Chapter 4 is largely based on the following publication:
Chapter 5

Clusters of cells in a hybrid E/M phenotype

5.1 Introduction: Interplay of Notch and EMT pathways

From an intracellular signaling perspective, the decision whether cells undergo no EMT, a partial EMT, or a complete EMT has received much theoretical and experimental attention (97, 147–157). However, often, these cell-fate decisions are influenced by non-cell autonomous factors such as ECM density and stiffness, and cell-cell communication with stromal cells (105, 189, 224–227). Notch signaling pathway serves as a hub of cell-cell communication both among the cancer cells as well as between cancer and stroma (189, 225, 228). Also, owing to the established role of Notch signaling in collective migration during developmental (type I) and tissue repair (type II) EMT (229, 230) and its fundamental role in spatial pattern formation in multiple developmental cases (231), Notch signaling can be expected to be of crucial importance in the formation of clusters
of Circulating Tumor Cells that migrate collectively. Here, we devise a specific theoretical framework to couple EMT/MET regulation with the Notch signaling pathway.

Notch pathway can be activated when the transmembrane receptor of one cell – Notch – interacts with the transmembrane ligand of another cell – Delta or Jagged, leading to the cleavage of Notch to produce Notch Intracellular Domain (NICD) (trans-activation) that translocates to the nucleus and controls the expression of many target genes of the Notch pathway, including Delta and Jagged (232). It inhibits Delta (233) but activates Jagged (234). Consequently, Notch-Delta (N-D) signaling forms a mutually inhibitory feedback loop between two adjacent cells, driving them to opposite fates – Sender (high ligand (Delta), low receptor (Notch)) and Receiver (low ligand (Delta), high receptor (Notch)) (Figure 5.1A). Conversely, Notch-Jagged (N-J) signaling forms a double positive feedback loop between the two adjacent cells and drives both cells to adopt a similar fate – hybrid Sender/Receiver (high ligand (Jagged), high receptor (Notch)) (Figure 5.1B) (231, 235, 236).

Notch and EMT circuits are intricately interconnected – NICD activates SNAIL (237, 238), miR-200 inhibits Jagged (239), and miR-34 represses Notch and Delta (240, 241). These inter-connections call for a detailed investigation to elucidate how cell-cell communication affects EMT/MET and consequently the spatial organization of E, hybrid E/M and M cells (Figure 5.1A).
Figure 5.1: Overview of the intracellular interplay between Notch signalling pathway and EMT circuit and Notch signalling tissue patterning outcomes. (a) Notch signalling is activated by the interaction of the transmembrane Notch receptor with the transmembrane ligand (Delta or Jagged) of a neighbouring cell. This trans-interaction cleaves Notch and causes the release of Notch intracellular domain (NICD) into the cytoplasm. NICD then enters the nucleus where it modulates the transcription of many target genes—it activates Notch, Jagged and Snail, and inhibits Delta. Glycosylation of Notch receptor by Fringe increases the affinity of Notch to bind to Delta and reduces that to Jagged (242, 243). Both the microRNAs miR-34 and miR-200 translationally inhibit proteins of the Notch pathway—miR-200 inhibits Jagged, and miR-34 inhibits both Notch and Delta. EMT-inducing signals (I_{ext}) such as Wnt and TGFβ can induce EMT by activating Snail. (b) Notch-Delta signalling creates an intercellular toggle switch leading neighbouring cells to adopt alternate fates—Sender cell (low Notch (receptor), high Delta (ligand)) and Receiver cell (high Notch (receptor), low Delta (ligand)), giving rise to a checkerboard-like pattern (lateral inhibition). (c) Notch-Jagged signalling creates an intercellular double positive feedback loop leading neighbouring cells to adopt similar fates (high Notch (receptor), high Jagged (ligand)), thereby propagating or inducing the same fate across the tissue (lateral induction).

5.2 Theoretical framework for coupled Notch-EMT dynamics

We devised a theoretical framework that couples Notch-Delta-Jagged (NDJ) signaling (235, 236) with EMT/MET regulation (97). The theoretical framework for NDJ
signaling is generalized from a previous theoretical framework to investigate Notch-Delta dynamics (244). The set of equations and the values of parameters used in the model are given in Appendix 4.

5.3 Results

5.3.1 EMT-inducing signals can activate Notch pathway

As the initial step towards deciphering the interplay between the core EMT circuit and the Notch circuit, we evaluated how the levels of ligands Delta and Jagged are affected in an individual cell (i.e. without any coupling to its neighbors via Notch) exposed to an EMT-inducing signal (I_{ext}). Increasing levels of I_{ext} decrease the levels of miR-34 and miR-200 (Figure S4.1), hence inducing a partial or complete EMT and relieving the repression on the levels of ligands Delta and Jagged (Figure 5.2A,B). Thus, an EMT-inducing signal can increase the levels of Notch ligands in a cell that can activate Notch signaling in its neighbors.

Next, we simulated the case of a two-dimensional layer of 50\times50 cells that communicate with one another via Notch-Delta-Jagged signaling, and measured the average levels of NICD in response to I_{ext}. Increasing values of I_{ext} led to increased NICD overall (Figure 5.2C). This model prediction was experimentally validated in breast epithelial cells MCF10A treated with TGFβ1 (a well-known EMT inducer) that displayed higher levels of NICD as compared to the control (Figure 5.2D).
Figure 5.2: Activation of Notch pathway via EMT inducer signal ($I_{ext}$). Bifurcation curves of the levels of (a) Delta and (b) Jagged as a function of EMT inducer levels ($I_{ext}$), for a one-cell system in the absence of external ligands ($D_{ext} = J_{ext} = 0$, $N_{ext} = 5000$). Increasing $I_{ext}$ induces a partial or complete EMT and concomitant increase in levels of Jagged and Delta. EMT phenotypes are defined based on the levels of miR200, miR34, Snail and Zeb, presented in figure S4.2. (c) Relative average levels of NICD (I) for a simulated two-dimensional layer of 50 × 50 cells for different levels of $I_{ext}$. The cells were simulated in a hexagonal lattice, starting from random initial conditions and the levels of NICD were measured after 120 h. (d) Immunofluorescence images of NICD (green) and cell nuclei (blue) for MCF10A cells treated with 5 ng/ml TGF-β1 for 6 days. Experiments in (d) performed by Mika Pietila in Dr. Mani’s group at the University of Texas MD Anderson Cancer Center, Houston, TX.

5.3.2 Notch signaling via both Delta and Jagged ligands can induce EMT

Next, to decipher how activating Notch signaling affects the core EMT circuit, we simulated the dynamics of the coupled EMT-Notch circuit in response to external ligands
Jext and Dext that represent the concentration of Delta and Jagged on the neighboring cells. Increase in either Jext or Dext can enhance the levels of NICD and induce an EMT through the activation of SNAIL by NICD (Figure 5.3A-C). Interestingly, for low levels of Jext, epithelial cells can attain one of the two phenotypes – (high Delta, low Notch) or (low Delta, high Notch), i.e. a Sender or Receiver of Notch signaling respectively (Figure 5.3B, C). However, when cells undergo a partial or complete EMT, the strong repression of Jagged by miR-200 is relieved, thereby enabling higher levels of both Jagged and NICD (Figure 5.3D). Consequently, cells can adopt a hybrid Sender/Receiver (S/R) phenotype that can induce the same fate as theirs in their neighbors (lateral induction) (235, 245). Thus, we hypothesize that Notch-Jagged, but not Notch-Delta, is likely to form the clusters of hybrid E/M and/or fully M cells.
Figure 5.3: Bifurcation curves, nullcline and phase diagram characterizing the response to external ligands. (a) Bifurcation curve of the levels of miR-200 as a function of the number of external Jagged (Jext) for Dext = 0 and Next = 5000 molecules. At low Jext, the cell adopts the epithelial (E) phenotype where it can be either a Sender (S) (high Delta, low Notch) or Receiver (R) (low Delta, high Notch)—(E),(S) or (E),(R). At increased levels of Jext, cell undergoes a transition to the hybrid epithelial/mesenchymal (E/M) phenotype. In this state, the cell presents high levels of both Notch and Jagged (Figure S4.2), therefore adopting a hybrid Sender/Receiver (S/R) state—(E/M), (S/R). Further increase in the levels of Jext induces a complete EMT and the cells adopt the mesenchymal (M) phenotype and also the S/R state—(M), (S/R). (b) Nullclines for the case of low levels of Jext (Jext = 600, Dext = 0, Next = 5000 molecules). The cell is in an epithelial phenotype, and can be either a Sender (high Delta, low Notch) or Receiver (low Delta, high Notch). Blue nullcline is for the condition of all ODEs being set to zero except for dD/dt and green nullcline is for the condition of all ODEs being set to zero except for dN/dt. Unfilled circles represent unstable steady states, whereas red filled circles represent the two stable states: Sender (high Delta, low Notch) and Receiver (low Delta, high Notch). (c) Bifurcation curve of the levels of miR-200 as a function of the number of external Delta (Dext) for Jext = 0 and Next = 5000 molecules. Green rectangle represents the range of parameter for the existence of Epithelial-Sender (E-S) phenotype. (d) Two-parameter bifurcation diagram (phase diagram) as a function of external Delta (Dext) and external Jagged (Jext). Each colour represents a different state: (E), (S) (dark green), (E),(R) (light green), (E/M),(S/R) (yellow) and (M),(S/R) (red).

Overall, both the ligands Delta and Jagged can induce EMT in a cell by activating the Notch pathway, but signaling via Notch-Delta vs. Notch-Jagged is expected to produce different spatial patterns of E, hybrid E/M and M cells.

5.3.3 Jagged-dominated signaling can lead to clusters of hybrid E/M cells

To characterize the differences in spatial patterning of E, hybrid E/M and M cells when EMT is induced via Notch-Delta signaling vs. Notch-Jagged signaling, we simulated a layer of 50*50 epithelial cells interacting via Notch signaling and evaluated
the dynamics of the coupled Notch-EMT circuit at a tissue level. The initial configuration of cells was chosen randomly (Figure S4.3) and to mimic both Delta-dominated and Jagged-dominated signaling, the simulations were done for many different levels of the production rates of Delta and Jagged. At low levels of production rates of both Delta and Jagged, no induction of EMT is observed. Higher production rates of either ligand increases the population of hybrid E/M and M cells, i.e. cells that undergo a partial or complete EMT (Figure 5.4 A, B).

When Notch-Jagged signaling dominates, most cells in the hybrid E/M phenotype tend to be adjacent to one another, but when Notch-Delta signaling dominates, these cells are usually spatially segregated (Figure 5.4C, D). Therefore, Notch-Jagged signaling, but not Notch-Delta signaling, can give rise to clusters of cells exhibiting a hybrid E/M phenotype. However, such cluster formation can be transient in absence of an EMT inducing signal because cells might revert to being epithelial (Figure S4.4).

These clusters can be stabilized in the presence of an external signal that either induces EMT or activates Notch signaling. An external signal that activates EMT-TF SNAIL (J_{ext}) increases the number of cells in a hybrid E/M or mesenchymal phenotype, both for Delta-dominated and Jagged-dominated signaling (Figure 5.5A, B, S4.5A,B). Jagged-dominated signaling predominantly drives cluster formation of hybrid E/M and mesenchymal cells, whereas Delta-dominated signaling results in ‘salt-and-pepper’ formation of epithelial and mesenchymal cells.

Because Notch signaling can also be activated in a paracrine manner (i.e. ligands secreted by other cells), we further evaluate the effect of soluble Delta and Jagged on
spatial arrangement of E, hybrid E/M and M cells. Higher levels of soluble Jagged increases the number of cells in a hybrid E/M phenotype but not those that are completely mesenchymal (Figure 5.5C, D, S4.5C, D) Therefore, the clusters are mostly composed of hybrid E/M cells. Similar behavior is observed under the influence of soluble Delta (Figure S4.6).

Although canonical Notch-Delta and Notch-Jagged signaling lead to different patterns, we observed that both soluble Delta and soluble Jagged affect the formation of cell clusters similarly. These differences can be attributed to the difference in dynamics of paracrine vs. juxtacrine signaling between Notch and its ligands. When the soluble ligand bind to Notch in a ‘target’ cell, NICD of that cell is cleaved and thus, levels of Jagged are upregulated but those of Delta get inhibited in that ‘target’ cells. Hence, irrespective of which ligand Notch bound to, the ‘target’ cell is likely to display (high Notch, high Jagged, low Delta) levels, a signature commensurate with hybrid E/M phenotype. Moreover, due to the ability to laterally induce their fate, most ‘target’ cells of soluble ligands of Notch signaling behave as hybrid Sender/Receivers.

Overall, Jagged-dominated Notch signaling enables the formation of clusters of hybrid E/M cells. However, the presence of Fringe, a glycosyltransferase that increases the binding affinity of Notch to bind to Delta and decreases that for Jagged, restricts the cluster formation (Figure S4.7). The modulation of cluster formation by Fringe is a typical example of how asymmetric modification of transmembrane ligand-receptor pairs can alter tissue patterning (246).
Figure 5.4: Tissue patterning for Delta-dominated and Jagged-dominated Notch signalling. Simulation of a two-dimensional layer of 50 × 50 cells interacting via Notch signalling. Fraction of cells for each phenotype: epithelial (E), epithelial/mesenchymal (E/M) and mesenchymal (M) for different production rates of (a) Delta and (b) Jagged. (c) Snapshot of the simulated tissue representing the spatial distribution of E, E/M and M cells for gD = 70 and gJ = 20 molecules h−1. (d) Same as (c) for gD = 20 and gJ = 70 molecules h−1. The initial levels of the proteins for each cell are initially chosen randomly (Figure S4.3) and the values are measured after an equilibrium time of 120 h. In the absence of any other external signal, the clusters of E/M are transient and disappear after 240 h (Figure S4.4).
Figure 5.5: Effect of external inducers of the Notch-EMT coupled circuit on tissue patterning. Simulation of a two-dimensional layer of 50 × 50 cells interacting via Notch-Delta-Jagged signalling. (a) Fraction of cells for each phenotype: epithelial (E), epithelial/mesenchymal (E/M) and mesenchymal (M) for different levels of an EMT-inducer signal ($I_{ext}$). (b) Snapshot of the simulated tissue representing the spatial distribution of E, E/M and M cells for $I_{ext} = 70$ molecules, gD = 20 molecules/hand gJ = 70 molecules/h. A majority of the cells that undergo EMT adopt the M state. (c) Fraction of cells for each phenotype for different levels of external soluble Jagged ($sJ_{ext}$) for $I_{ext} = 0$ molecules, gD = 20 molecules/h and gJ = 70 molecules/ h. (d) Snapshot of the simulated tissue representing the spatial distribution of E, E/M and M cells $sJ_{ext} = 4000$ molecules. The levels were measured after 120 h, starting from the configuration presented in figure 5.4c.
Finally, we investigated how the spatial patterning of E, hybrid E/M and M cells is modulated by Delta-dominated and Jagged-dominated Notch signaling when cells are in a partial or complete EMT state to begin with. The effects of Delta-dominated vs. Jagged-dominated signaling are strikingly different – whereas in the case of Delta-dominated signaling, cells undergo an MET and adopt an epithelial phenotype, MET rarely happens in the case of Jagged-dominated signaling. Consequently, in the case of Delta-dominated signaling, many epithelial and non-epithelial cells arrange to form ‘salt-and-pepper’ patterns (Figure S4.8), but in the case of Jagged-dominated signaling, the initial random pattern of E/M and M cells reorganizes to form clusters of hybrid E/M cells (Figure 5.6, S4.9). These clusters can further be stabilized by lateral induction, thus Notch-Jagged signaling can both induce and maintain clusters of hybrid E/M cells. In other words, Notch-Jagged signaling can act as a ‘phenotypic stability factor’ for the hybrid E/M phenotype.

Figure 5.6: Notch-Jagged signalling acts as a ‘phenotypic stability factor’ for the hybrid E/M phenotype. Simulation of 50 × 50 cells interacting via N-D-J signalling. (b) Fraction of cells adopting epithelial (E), epithelial/mesenchymal (E/M) and mesenchymal (M) phenotypes at different time points for the given initial condition. (a, c) Levels of miR200 for 50 × 50 hexagonal lattice at t = 0 and t = 360 h. Red cells are in an M phenotype, yellow ones in a hybrid E/M one.
5.3.4 Role of Jagged-dominated signaling in mediating chemoresistance

Motivated by our modeling predictions that ‘phenotypic stability factors’ such as OVOL and GRHL2 can associate a hybrid E/M phenotype with a higher tumor-initiation potential (100, 186), we investigated whether Notch-Jagged signaling is active in breast cancer cells driving tumor-initiation potential and chemoresistance. Cells co-expressing an epithelial marker (CD24) and a mesenchymal marker (CD44) and other canonical epithelial and mesenchymal genes – CD24$^\text{hi}$ CD44$^\text{hi}$ – have been shown to possess adaptive chemoresistance and high tumor-initiation in vitro and in vivo (44, 247). Therefore, we investigated the levels of NICD (cleaved Notch denoting active Notch signaling) and Jagged1 in different subpopulations of MDA-MB-231 – CD24$^\text{lo}$ CD44$^\text{hi}$ and CD24$^\text{hi}$ CD44$^\text{hi}$. Cells in a hybrid E/M phenotype (CD24$^\text{hi}$ CD44$^\text{hi}$) express higher levels of NICD than the mesenchymal CD24$^\text{lo}$ CD44$^\text{hi}$ cells (Figure 5.7A). Next, these breast cancer cells were treated with a cytotoxic chemo-therapeutic used in the first-line treatment of triple negative breast cancer (TNBC) – docetaxel – followed by drug washout, substrate reattachment, and acute population outgrowth, resulting a population of drug-tolerant cells (DTCs) (Figure 5.7B).

Consistent with earlier reports (247), we confirmed that the DTCs have higher expression of both CD24 (epithelial marker) and CD44 (mesenchymal marker) as compared to parent population, hence indicating a shift towards a hybrid E/M phenotype (Figure 5.7 C, D). Intriguingly, these DTCs express higher amount of NICD and Jagged, but lower levels of Delta as compared to the parent population (Figure 5.7E). These results are consistent with enhanced JAG1 levels and suppressed Delta levels in drug-
resistant small-cell lung cancer cells H69AR as compared to H69, the parent population (248), short-term treatment therapies enriching for JAG1-NOTCH4 signaling mediated breast CSCs (249), acquired chemoresistance via JAG1-NOTCH1 signaling in ovarian cancer (250), and CD24\textsuperscript{hi} CD44\textsuperscript{hi} marking pancreatic and gastric CSCs (251, 252). Furthermore, CD24\textsuperscript{hi} CD44\textsuperscript{hi} expression overlaps with high levels of P-cadherin (CDH3), another proposed marker for a hybrid E/M phenotype (168). Put together, these observations support the hypothesis that Jagged-dominated Notch signaling may be instrumental in maintaining a hybrid E/M phenotype as well as associating them with a higher likelihood of gaining ‘stemness’, as characterized by enhanced drug resistance and tumor-initiation potential.

![Diagram showing CD44, CD24, and NICD expression in MDA-MB-231 cells under various conditions.](image)
Figure 5.7: Drug-tolerant population exhibits enhanced levels of Notch-Jagged signaling (a) Representative confocal microscopy shows CD44, CD24 and cleaved notch (NICD) in a population of drug naive MDA-MB-231. Yellow arrows indicate CD44$^{Hi}$CD24$^{Lo}$ (M) population of cells and the white arrows indicate the CD44$^{Hi}$CD24$^{Hi}$ (E/M) cells. Histogram (right panel) shows quantification of NICD in the distinct phenotype populations (M versus E/M). N = 3 biological replicates. (b) Schematic describes the experimental protocol to generate drug-tolerant cells (DTCs) parental MDA-MB-231 cells were treated with docetaxel at 100 nM (20× the IC50) and subsequently selected by substrate re-attachment and acute population outgrowth. (c) Representative confocal microscopy shows CD44, CD24 and NICD in the MDA-MB-231 parent and DTC populations. Right panel shows quantification of fluorescence intensity of each signal determined by at least 25 individual fields. N = 3 biological replicates. (d) Representative confocal microscopy shows Jagged and Delta expression in MDA-MB-231 parent and DTC. DAPI nuclear stain (blue). N = 3 biological replicates. In vitro experiments performed by Aaron Goldman at Dr. Sengupta’s group at Department of Medicine, Harvard Medical School, Boston, MA.

5.4 Discussion

Here, we elucidate how cell-cell communication through Notch signaling can be crucial in forming clusters of cells in a hybrid E/M phenotype. Our results indicated that while Notch signaling can induce EMT via both of its ligands – Delta and Jagged, Jagged-dominated, but not Delta-dominated Notch signaling can drive the formation of clusters. The formation and stability of these clusters is enhanced by EMT-inducing signals and/or soluble ligands of the Notch signaling pathway, both of which can promote Jagged-dominated signaling between the adjacent cells. Since Notch-Jagged signaling is usually associated with lateral induction (i.e. driving their neighbors to the same fate as theirs) (235, 236, 245), Notch-Jagged signaling between neighbors can stabilize the ‘metastable’ hybrid E/M phenotype and therefore enable clusters of CTCs – the real ‘bad
actors’ of metastasis (9). Recent analysis of CTC clusters as well as clusters of locally disseminated tumor cells suggested that these clusters were enriched for K14+ cells, and JAG1 was one of the top 4 genes that correlated with K14. K14 knockdown reduced the mean number of metastasis 7-fold, thereby supporting our hypothesis about the critical role of JAG1 in CTC cluster formation (79).

Therapeutic targeting of JAG1 is not only expected to potentially ‘break’ the clusters of CTCs, but also allay their chemoresistance and/or tumor-initiation potential. Recent studies indicate that cells in a hybrid E/M phenotype (identified by CD24\textsuperscript{hi} CD44\textsuperscript{hi} here) can form much more tumors as compared to those in a mesenchymal phenotype (44), and can display resistance to multiple drugs such as cisplatin, paclitaxel and salinomycin (159). Our experimental data showing that the drug-tolerant population of MDA-MB-231 cells bears CD24\textsuperscript{hi} CD44\textsuperscript{hi} expression (247) and displays Jagged-dominated Notch signaling suggest that Notch-Jagged signaling may behave as intercellular ‘phenotypic stability factor’ (162) for hybrid E/M phenotype. Furthermore, it is consistent with the implications of Notch-Jagged signaling in maintaining CSC population and chemoresistance (225, 228, 253), aggressive tumor progression(254), and reinforces the emerging notion that the CSCs lie mid-way on the ‘EMT axis’ (9, 43, 88, 159, 186, 255).

As indicated earlier in Chapters 2, 3, and 4, a hybrid E/M phenotype and clusters of CTCs are much better poised to initiate secondary tumors or metastases. We predict that targeting Jagged1 (JAG1) therapeutically might mitigate these advantages and significantly curtail the metastatic load. This prediction is consistent with recent reports
in breast cancer illustrating that strong JAG1 staining intensity correlates with metastatic relapse, lymph node positivity, and higher number of disseminated tumor cells in bone marrow aspirates. Furthermore, more than 85% of CTCs expressed JAG1, and the presence of JAG-positive CTCs significantly correlates with shorter progression-free survival (256). Besides, targeting JAG1 can also mollify the tumor promoting effect of inflammatory cytokines that activate JAG1 and/or inhibit Delta, enhancing Notch-Jagged signaling (257, 258). Therefore, targeting JAG1 specifically, as recently attempted via decoys (259), can be of paramount importance in halting aggressive tumor progression (254), and can mitigate the side effects of inhibiting Notch pathway entirely (260).

To the best of our knowledge, ours is the first theoretical study elucidating how any form of intercellular affects the intracellular regulation of EMT. While future experimental work remains to be done both to validate our predicted spatial correlation of E, E/M and M cells, and the causal role of Notch-Jagged signaling in mediating tumor-initiation potential and/or drug resistance, our theoretical framework proposes a critical therapeutic target – JAG1. Our framework can be further used to investigate (a) how external factors such as inflammation affect the formation of clusters of CTCs, (b) how are different subpopulations of CSCs spatially located in a tumor mass (218), and (c) how cancer cell colonies can cooperate among themselves driving tumor progression (1, 261).

**Note:** Chapter 5 is largely based on the following publication:

Chapter 6

Summary

This thesis characterizes the stability and tumor-initiation potential of a hybrid epithelial/ mesenchymal (E/M) phenotype and elucidates its implications in driving cancer metastasis. Using an integrated theoretical and experimental approach, the work presented here strongly suggests that despite tacit assumptions, a hybrid E/M phenotype need not always be metastable or transient. Instead, as demonstrated by H1975 lung cancer cells, a hybrid E/M phenotype can be stably maintained in culture, and knockdown of predicted ‘phenotypic stability factors’ (PSFs) GRHL2 and OVOL2 can destabilize the hybrid E/M phenotype (100). Besides, cell-cell communication via Notch-Jagged signaling can contribute to maintain cells in a hybrid E/M phenotype and form clusters of Circulating Tumor Cells (CTCs) containing hybrid E/M cells (102). Enhanced Notch-Jagged signaling in drug-tolerant MDA-MB-231 cells(102), and higher levels of PSFs such as OVOL1/2 and GRHL2 correlating with poor patient survival in
multiple tumor types (100) indicate that a stable hybrid E/M phenotype can aggravate tumor progression (Figure 6.1).

**Figure 6.1: Interplay among hybrid E/M phenotype, high tumor-initiation potential, Notch-Jagged signaling, and drug/therapy resistance**: Partial EMT can correlate with clustered migration (Chapters 2, 3), high tumor-initial potential (Chapter 4) and can be stabilized by Notch-Jagged signaling that can mediate drug resistance too (Chapter 5).

Recent observations in cell lines (25, 43, 44, 152), primary and metastatic tumors (18, 49, 60), mouse models (61, 62) and bloodstream of breast, lung and prostate cancer patients (19, 58, 59) have identified cells co-expressing epithelial and mesenchymal markers. However, most, if not all, of these studies represent a temporal snapshot. Moreover, many reports on partial EMT have involved only ensemble measurements (38, 42); the population labelled as ‘hybrid E/M’ can contain either mixed subpopulations of E and M cells (H2291 - lung cancer), and/or truly hybrid E/M (H1975) cells (42, 100). Therefore, future experiments on the role of cellular plasticity in tumor progression should both investigate the temporal stability of a hybrid E/M phenotype and delineate the potential differences in invasive and metastatic traits of a single-cell hybrid E/M
subpopulation vs. admixtures of E and M cells. Various recently developed intracellular reporters (262, 263) can play a crucial role in addressing these fundamental questions.

While we expect a conceptual correspondence between a hybrid E/M phenotype and collective cell migration based on the observations in H1975 cells, mammary morphogenesis, and wound healing (24, 35, 100), the coupling between genetics and biophysics of EMT is poorly understood and needs dire attention both from theoretical and experimental perspectives. Thus, the gene expression pattern need not always exclude motility for epithelial cells (264). Similarly, neither all CTC clusters necessarily contain hybrid E/M cells nor all hybrid E/M cells form clusters (19, 86, 87). Moreover, the set of morphological, migratory, and lineage-associated changes that are referred to as EMT is highly likely to be context-dependent (265); therefore, the labelling of a phenotype as partial EMT or a hybrid E/M should consider this inherent contextual heterogeneity. This consideration would also be of paramount importance in identifying the various intermediate state(s) of EMT, their morphology, gene expression and/or proteomic profile, and relative stability.

Identifying and characterizing intermediate state(s) of EMT can help appreciate the interplay between tumor and its microenvironment in a more nuanced manner. For instance, integrating the theoretical framework developed here with those capturing stromal cell plasticity mechanisms can offer mechanistic insights into an association between accumulation of FOXP3+ T-cells and M2-macrophages with tumor budding (266), a clinical manifestation of partial EMT (85). Besides, integrating these mechanistic intra- and inter-cellular networks with population dynamics of various subpopulations of
cancer cells such as metastasis-initiating cells (MICs) (5, 190–192, 267, 268) can be crucial to address unanswered questions about metastatic growth and dormancy.

From a clinical standpoint, this study recommends isolating clusters of Circulating Tumor Cells (CTCs) in addition to the individually migrating CTCs. With the first liquid biopsy being approved by the FDA recently and novel technologies being developed to capture the CTC clusters (77), analyzing the frequency of clusters vs. CTCs and/or the cluster size distribution can yield novel insights into stratifying patient risk and monitoring disease progression.

Overall, by elucidating the implications of a hybrid epithelial/mesenchymal phenotype in cancer metastasis, this thesis extols the crucial role that fine-tuned cellular plasticity can play in tumor progression by helping cancer cells adapt to their ever changing microenvironment. Meanwhile, multiple other examples have been explored in both physiological and pathological contexts where cells need not undergo ‘all-or-none’ transitions; rather they can adopt intermediate phenotype(s) (269–273). Investigating the stability, plasticity, and the contribution of these phenotypes to homeostasis and/or malignancy is expected to provide a stimulating and fertile ground for integrating theory and experiment in molecular and cellular biology, especially oncology. Such an integration of ‘bottom-up’ mechanistic modeling with quantitative experiments, with able support by ‘top-down’ models to infer relevant correlations in multi-dimensional and clinical datasets, is expected to take a major step forward towards establishing “cancer research as an increasingly logical science, in which myriad phenotypic complexities are manifestations of a small set of underlying organizing principles”(117).
Supplementary Information

Appendix 1: Tristability in the core EMT/MET network

Theoretical framework for microRNA-based chimera circuits (MBC)

(a)

(b)

<table>
<thead>
<tr>
<th>Population</th>
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<th>Effective Translation</th>
<th>mRNA Active Degradation</th>
<th>microRNA Active Degradation</th>
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<td>$I_0$</td>
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<td>0</td>
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<td>$C^1_0$</td>
<td>$I_1$</td>
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<td>$\gamma_{\mu_1}$</td>
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<tr>
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<td>$I_2$</td>
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<td>\vdots</td>
<td>\vdots</td>
<td>\vdots</td>
<td>\vdots</td>
</tr>
<tr>
<td>$M^n_0(\mu)$</td>
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<td>$I_n$</td>
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<tr>
<td>Total</td>
<td></td>
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</tbody>
</table>
**Figure S1.1: Schematic diagram of MBC model** (a) Illustration of the MBC model formulation, similar to Fig. 2.2 in the main text but also including transcriptional regulation explicitly. (b) Quantitative description of miR-mediated translation silencing. The leftmost column shows the illustrations of various miR-mRNA complexes. In $i$th row, $i-1$ binding sites are occupied by miR. The second column to the left shows the population of different configurations in the condition of binding/unbinding equilibrium. The third column shows the degeneracy (number of possible cases) for each configuration. The fourth to sixth columns show the notations for the individual translation rate, active mRNA degradation, and active miR degradation. The overall effects to all configurations are listed in the last row. Active degradation refers to degradation of miR or mRNA during the miR-mRNA complex formation, and is distinct from their innate degradation rates.

**Equations and parameters for miR-34/SNAIL/miR-200/ZEB circuit**

The parameters in these equations were estimated largely from existing experimental data. The innate degradation rates for the proteins, mRNAs and miRs were selected according to the half-lives of each molecule from the experimental data. Since the typical half-life of mammalian proteins is about 10 hours (274), we chose 0.1 hour$^{-1}$ as the degradation rate for ZEB, and 0.125 hour$^{-1}$ for SNAIL. That of SNAIL is larger, because SNAIL is less stable (148). The half-lives of mRNA is roughly a few hours (140), so we chose 0.5 hour$^{-1}$ as the innate degradation rate for mRNA of both ZEB and SNAIL. As miR is generally more stable than mRNA (141, 142), the innate degradation rate of miR for both miR-200 and miR-34 were selected as 0.05 hour$^{-1}$.

The expression levels for various molecules were estimated according to the usual protein concentration and cell dimension for eukaryotic cells, and were converted to the number of molecules. The length scale of a typical eukaryotic cell is about 10µm, and the characteristic concentration for a signaling protein is about 10nM to 1µM (275) So for a 1µM protein, the number of proteins is \[ \frac{6 \times 10^{23}}{10^3} \times 10^6 \times (10^{-6})^3 \] which is roughly one million molecules. Moreover, the ratio of protein/mRNA numbers for a gene is about 2800 (276), therefore the number of mRNA for a gene should be around 1000 molecules. The number of molecules for a microRNA should be around 10,000 (277). In this study, we used “K molecules” as the unit for the number of molecules for proteins,
mRNAs and miRs. The transcription rates for different molecules were chosen accordingly, so that the molecule levels are consistent with aforementioned expectations. The translation rate for a gene is about 140 proteins per mRNA per hour (276), so we used 0.1K proteins per mRNA per hour as the translational rate. Besides, in transcriptional regulation, the changes in synthesis rates were set to be about five to ten fold, i.e. for activator is from 5 to 10, and corresponding to repressor is from 0.1 to 0.2. Parameters are listed in Tables S1, S2.

(a) Parameters for the MBC model

<table>
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<tr>
<th></th>
<th>L Only</th>
<th>Y Only</th>
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<th>L &gt; Y</th>
<th>L &lt; Y</th>
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<td>2.1K</td>
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<td>200K</td>
<td>300K</td>
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<td>50K</td>
<td>30K</td>
<td>25K</td>
<td>50K</td>
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</table>

(b) Parameters for the miR-200/ZEB circuit

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</thead>
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<td>200K</td>
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<tr>
<td>$n_{S,mZ}$</td>
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<td>10K</td>
</tr>
</tbody>
</table>

(c) Parameters for the miR-34/SNAIL circuit

<table>
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<th>Molecules/ Hour</th>
</tr>
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<tr>
<td>$n_{S,mS}$</td>
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<td>200K</td>
<td>1.35K</td>
</tr>
<tr>
<td>$n_{Z,34}$</td>
<td>2</td>
<td>600K</td>
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</tr>
<tr>
<td>$n_{T}$</td>
<td>2</td>
<td>10K</td>
<td></td>
</tr>
</tbody>
</table>

Table S1.1: List of parameters used in the simulation. The parameters highlighted in color are the ones used for model calculations. Other parameters in (a) are parameters used to test multistability of the miR-200/ZEB circuit with varying contributions of different aspects of miR-mediated regulation (as shown in SI Fig 2).
Table S1.2: Parameters for $l_i, Y_{mi}, Y_{\mu}$ for various cases. ‘L only’ refers to the case when only translational inhibition is considered, ‘Y only’ refers to when only active degradation of mRNA is considered. ‘Both’ denote the cases when both these functions are incorporated, but with different relative weights. The parameters chosen here are such that the total silencing for each case is similar to available experimental data (Figure S5). The parameters highlighted in blue are the ones eventually used in our modeling calculations.

Table S1.3: Parameters varied to explore the effect of ZEB and SNAIL autoregulation. Parameters highlighted in green represent the case without self-inhibition of SNAIL (Fig S1.3), those highlighted in orange are for Fig S1.4a, in blue for Fig S1.4b, in purple for Fig S1.4c, d.

We tested five combinations for the MBC model (Table S2) of the (miR-200/ZEB) circuit to assess the effect of different relative strengths of translational repression and active miRNA degradation. When only one silencing mechanism was present, we could attain at most bistability (Fig S2 b, c). By slightly adjusting other parameters to vary relative strengths of these two silencing mechanisms, we found tristability, but the three stable steady states were best found to characterized when the translational inhibitory mechanism has a dominant role to play (Fig S2 d-f). Hence,
different silencing mechanisms could affect the multistability of miR-based chimeric toggle switches. We also found that the cooperative self-activation of ZEB is essential for tristability of miR-200/ZEB module and the combined circuit. Reducing the Hill coefficient of ZEB self-activation to 0 or 1 for either the miR-200/ZEB circuit or combined circuit render the circuit to be only bistable. Also, self-inhibition of SNAIL does not render miR-34/SNAIL to be bistable (Figs S3, S4, Table S3).

**Figure S1.2: Stable states of (miR-200/ZEB) circuit for different parameters of MBC model.** (a) Illustration of (miR-200/ZEB) circuit, with SNAIL as the external input signal. (b)-(f) show nullclines of the system for different parameter combinations as shown in Table S1. Red nullcline is for \( \frac{d\mu_{200}}{dt} = 0, \frac{dZ}{dt} = 0 \), blue nullcline is for \( \frac{dm_{Z}}{dt} = 0, \frac{dZ}{dt} = 0 \). Green solid points denote stable fixed points and green hollow circles denote unstable or saddle fixed points. Parameters are listed in Tables S1, S2.
Figure S1.3: Effect of self-inhibition of SNAIL on monostability of the circuit. Nullclines of miR-34/SNAIL module without the self-inhibition of SNAIL. Parameters used are given in Tables S1-3.

Figure S1.4: Effects of self-activation of ZEB on multistability of the circuit. Nullclines are plotted for the circuit without self-activation (a,b) and with Hill coefficient $= 1$ (c, d). Only the miR-200/ZEB module is analyzed in (a) and (c), and the combined network is analyzed in (b) and (d).
Experiments indicate that SNAIL protein levels reduce to about 80% of their baseline levels when miR-34 binds to one binding site on SNAIL mRNA, about 50% when it binds to both of them (137). Also, with 6 binding sites on ZEB mRNA, miR-200 brings down ZEB protein levels to 10% (147). Since different miRs may adopt different mechanisms for silencing, we tested 5 different versions, each represented by a distinct set of parameters. The total silencing effect, as shown in Fig S1a, is given by

\[ P(\mu) = \frac{L(\mu)}{1 + (Y_m(\mu)/k_m)} \]

For ‘L only’ mode, i.e. only translational inhibition considered, \( \gamma_m = 0 \), and \( P(\mu) = L(\mu) \). In next case, when only mRNA degradation is considered (‘Y only’), \( l_i = 0 \), and \( P(\mu) = \frac{1}{1 + (Y_m(\mu)/k_m)} \). In remaining cases, the relative weightage of translational inhibition and active mRNA degradation are varied such that they denote similar profiles for total silencing, as shown in Fig S5. These parameters were chosen to match the existing experimental data at \( \mu = \mu_0 \), i.e. the value of \( P(\mu) \) at \( \mu = \mu_0 \) is approximately 0.8, 0.5, 0.1 and 0.1 for 1, 2, 5, and 6 binding sites.
Figure S1.5: Magnitude of $P(\mu)$ for different sets of parameters. Every panel contains 5 curves, each corresponding to different cases as explained above and mentioned in Table S2. (a)-(d) denote cases for 1, 2, 5, 6 binding sites respectively. The vertical dotted line indicates the value of $P(\mu)$ at $\mu = \mu_0$, i.e. the miR threshold levels.

The effect of noise in external signal on miR-34/SNAIL

We simulated the dynamics of miR-34/SNAIL in presence of the external noise on the signal $I$. To simulate the external noise, signal $I$ follows the stochastic differential equation $\dot{I} = (I_0 - I) + \alpha(t)$ where $<(t), (t')> = (t, t)$. $I_0$ is set to be 50 K molecules, to be 0.04 Minute$^{-1}$, and to be 16 (K molecules/Minute)$^2$. So $\bar{I} = I_0 = 50$ K molecules, $\alpha = 1/(2\sigma^2) \sim 14$ K molecules, and $<I(0) I(t)> = \sigma^2 \exp(-t/\tau)$, $\tau = 1/\alpha = 25$ Minutes.

![Diagram](image-url)
**Figure S1.6: miR-34/SNAIL module serves as a filter for external noise.** The upper panel shows simulation results when the noisy signal is an input to miR-34. The upper left diagram illustrates the two simulations, one with SNAIL self-inhibition and one without. The upper right diagram shows the distribution of the SNAIL levels. The lower panel shows simulations when the noisy signal is a direct input to SNAIL. The lower left diagram illustrates the three simulations: one with SNAIL self-inhibition, one without self-inhibition, and one without both miR-34 and SNAIL self-inhibition. The lower right diagram shows the distribution of the SNAIL levels in these three cases.

We checked the variations in the SNAIL level in two cases – when noisy signal is taken as an input on (a) SNAIL and (b) miR-34. In both cases, self-inhibition of SNAIL makes the deviation of the SNAIL level smaller (black line). In absence of SNAIL self-inhibition, the deviation of the SNAIL level is similar, irrespective of the presence (red line) or absence (blue line) of miR-34 (Figure S1.6). Thus, the self-inhibition of SNAIL may serve as a filter of the external noise, and thus prevent the aberrant activation of EMT.
Appendix 2: Stability of the hybrid E/M phenotype

Theoretical framework to incorporate GRHL2, OVOL1/2, and miR-145

miR-200/ZEB/GRHL2 circuit

The miR-200/ZEB/GRHL2 circuit has five species - microRNA miR-200 (\(\mu_{200}\)), ZEB mRNA (\(m_z\)), ZEB protein (\(Z\)), GRHL2 mRNA (\(m_G\)), and GRHL2 protein (\(G\)). All of them have an innate production and degradation rate. Effects of miR-200 on ZEB are captured by both the degradation of mRNA by miRNAs (depicted by \(Y_{m}(\mu)\)) and inhibition of translation by miRNAs (depicted by \(L(\mu)\)). Also, the miRNAs that bind to mRNAs can be degraded after forming a complex with mRNAs (depicted by \(Y_{m}(\mu)\)). A detailed derivation of these functions is presented in our earlier work on devising a theoretical framework for microRNA-based circuits (97). GRHL2, a key regulator of morphogenesis (161), forms a mutually inhibitory loop with ZEB. Promoter region of GRHL2 has 3 binding sites for ZEB1, and that of ZEB1 has 1 binding site for GRHL2 (108, 109).

The dynamics of miR-200 (\(\mu_{200}\)) can be described by the following equation:

\[
\frac{d\mu_{200}}{dt} = g_{\mu_{200}} H^S(Z, \lambda_{Z, \mu_{200}}) H^S(S, \lambda_{S, \mu_{200}}) - m_z Y_{\mu}(\mu_{200}) - k_{\mu_{200}} \mu_{200} \cdots \cdots \ (1)
\]

where \(g_{\mu_{200}}\) and \(k_{\mu_{200}}\) are the innate production and degradation rates of miR-200 respectively. \(H^S(Z, \lambda_{Z, \mu_{200}})\) represents the transcriptional inhibition of miR-200 by ZEB and \(H^S(S, \lambda_{S, \mu_{200}})\) represents transcriptional inhibition of miR-200 by SNAIL. \(Y_{\mu}(\mu_{200})\) represents the degradation rate of miR-200 due to forming a complex with ZEB mRNAs.

The dynamics of ZEB mRNA (\(m_z\)) and ZEB protein (\(Z\)) are described by the following equations:
\[
\frac{dm_Z}{dt} = g_{m_Z} H_{\text{m}}(G, \lambda_{G,m_Z}) H_{\text{m}}(Z, \lambda_{Z,m_Z}) H_{\text{m}}(S, \lambda_{S,m_Z}) - m_Z Y_m(\mu_{200}) - k_{m_Z} m_Z \quad \cdots \quad (2)
\]

\[
\frac{dZ}{dt} = g_Z m_Z L(\mu_{200}) - k_Z Z \quad \cdots \quad (3)
\]

where \( g_{m_Z} \) and \( g_Z \) are the innate production rates of ZEB mRNA and ZEB protein respectively, and \( k_{m_Z} \) and \( k_Z \) are their respective innate degradation rates. \( H_{\text{m}}(Z, \lambda_{Z,m_Z}) \) denotes transcriptional self-activation of ZEB, and \( H_{\text{m}}(S, \lambda_{S,m_Z}) \) denotes transcriptional activation of ZEB by SNAIL. \( Y_m(\mu_{200}) \) represents the degradation of ZEB mRNA due to forming mRNA-miRNA complexes with miR-200, \( L(\mu_{200}) \) denotes the translational inhibition of ZEB by miR-200, and \( H_{\text{m}}(G, \lambda_{G,m_G}) \) represents the inhibition from GRHL2.

Dynamics of GRHL2 mRNA \( (m_G) \) and GRHL2 protein \( (G) \) are described by these equations:

\[
\frac{dm_G}{dt} = g_{m_G} H_{\text{m}}(Z, \lambda_{Z,m_G}) - k_{m_G} m_G \quad \cdots \quad (4)
\]

\[
\frac{dG}{dt} = g_G m_G - k_G G \quad \cdots \quad (5)
\]

where \( g_{m_G} \) and \( g_G \) are innate production rates of GRHL2 mRNA and protein respectively, and \( k_{m_G} \) and \( k_G \) are their respective degradation rates. \( H_{\text{m}}(Z, \lambda_{Z,m_G}) \) represents the transcriptional inhibition of GRHL2 by ZEB.

When considering an external activation \( (SA) \) or inhibition signal \( (SI) \) on GRHL2, shifted Hill functions \( H_{\text{m}}(SA, \lambda_{SA,m_G}) \) and \( H_{\text{m}}(SI, \lambda_{SI,m_G}) \) are respectively multiplied to the production term of GRHL2 mRNA:
\[
\frac{dm_{G}}{dt} = g_{m_{G}} H^{s^{-}}(Z, \lambda_{Z,m_{G}}) H^{s^{+}}(S, \lambda_{S,m_{G}}) - k_{m_{G}} m_{G} \quad \cdots \cdots \cdots (4)
\]

or
\[
\frac{dm_{G}}{dt} = g_{m_{G}} H^{s^{-}}(Z, \lambda_{Z,m_{G}}) H^{s^{+}}(SI, \lambda_{SI,m_{G}}) - k_{m_{G}} m_{G} \quad \cdots \cdots \cdots (4)
\]

**miR-200/ZEB/miR-145/OCT4 circuit**

miR-200/ZEB/miR-145/OCT4 circuit has six species - microRNA miR-200 \((\mu_{200})\), ZEB mRNA \((m_{Z})\), ZEB protein \((Z)\), miR-145 \((\mu_{145})\), OCT4 mRNA \((m_{O})\), and OCT4 protein \((O_{C})\). All of them have an innate production and degradation rate. The effects of miR-200 on ZEB are captured similarly to as in the miR-200/ZEB/GRHL2 circuit; but the effect of miR-145 on OCT4 and ZEB are represented by shifted Hill functions, because the silencing effect of microRNAs on their targets with a relatively smaller number of binding sites (~2) can be approximated as a Hill function \((97)\). miR-145 and OCT4 form a mutually inhibitory feedback loop, with one binding site of OCT4 on miR-145 promoter region, and one binding site of miR-145 on the 3’ UTR of OCT4 mRNA \((278)\). OCT4 can transcriptionally activate miR-200c via two binding sites \((198)\), and miR-145 inhibits ZEB via one binding site in its 3’ UTR, as predicted by TargetScan \((279)\). The siRNA-mediated depletion of ZEB increases miR-145 two-to-three fold \((280)\), so we assume two binding sites for the inhibition of miR-145 by ZEB. The dynamics of miR-200 \((\mu_{200})\) can be described by the following equation:

\[
\frac{d\mu_{200}}{dt} = g_{\mu_{200}} H^{s^{-}}(Z, \lambda_{Z,\mu_{200}}) H^{s^{-}}(S, \lambda_{S,\mu_{200}}) H^{s^{+}}(O_{C}, \lambda_{O_{C},\mu_{200}}) - m_{Z} Y_{\mu}(\mu_{200}) - k_{\mu_{200}} \mu_{200} \cdots \cdots \cdots (6)
\]

where \(g_{\mu_{200}}\) and \(k_{\mu_{200}}\) are the innate production and degradation rates of miR-200 respectively. \(H^{s^{-}}(Z, \lambda_{Z,\mu_{200}})\) represents the transcriptional inhibition of miR-200 by ZEB, \(H^{s^{-}}(S, \lambda_{S,\mu_{200}})\) represents the transcriptional inhibition of miR-200 by SNAIL, and \(H^{s^{+}}(O_{C}, \lambda_{O_{C},\mu_{200}})\) denotes the transcriptional activation of miR-200 by OCT4. \(Y(\mu_{200})\) represents the degradation rate of miR-200 due to forming a complex with ZEB mRNAs.
The dynamics of ZEB mRNA \((m_Z)\) and ZEB protein \((Z)\) are described by the following equations:

\[
\begin{align*}
\frac{dm_Z}{dt} &= g_{m_z} H^{S+}(\mu_{145}, \lambda_{m_z}, m_z) H^{S+}(Z, \lambda_{Z,m_z}) - m_Z Y_m(\mu_{200}) - k_{m_z} m_Z \quad \cdots \quad (7)\\
\frac{dZ}{dt} &= g_z m_Z L(\mu_{200}) - k_z Z \quad \cdots \quad (8)
\end{align*}
\]

where \(g_{m_z}\) and \(g_z\) are the innate production rates of ZEB mRNA and ZEB protein respectively, and \(k_{m_z}\) and \(k_z\) are their respective innate degradation rates. \(H^{S+}(Z, \lambda_{Z,m_z})\) denotes the transcriptional self-activation of ZEB. \(H^{S+}(S, \lambda_{S,m_z})\) denotes the activation of ZEB by SNAIL, and \(H^{S-}(\mu_{145}, \lambda_{a_{145}, m_z})\) denotes the inhibition of ZEB by miR-145. \(Y_m(\mu_{200})\) represents the degradation of ZEB mRNA due to forming mRNA-miRNA complexes with miR-200. \(L(\mu_{200})\) denotes the inhibition of ZEB by miR-200.

The dynamics of miR-145 \((\mu_{145})\), OCT4 mRNA \((m_{O_c})\), and OCT4 protein \((O_c)\) are given by:

\[
\begin{align*}
\frac{dm_{O_c}}{dt} &= g_{m_{O_c}} H^{S-}(\mu_{145}, \lambda_{m_{O_c}}, m_{O_c}) - k_{m_{O_c}} m_{O_c} \quad \cdots \quad (9)\\
\frac{dO_c}{dt} &= g_{O_c} m_{O_c} - k_{O_c} O_c \quad \cdots \quad (10)\\
\frac{d\mu_{145}}{dt} &= g_{\mu_{145}} H^{S-}(Z, \lambda_{Z,\mu_{145}}) H^{S-}(O_c, \lambda_{O_c, \mu_{145}}) - k_{\mu_{145}} \mu_{145} \quad \cdots \quad (11)
\end{align*}
\]

where \(g_{m_{O_c}}\), \(g_{O_c}\) and \(g_{\mu_{145}}\) represent the innate production rates for OCT4 mRNA, OCT4 protein and miR-145 respectively; and \(k_{m_{O_c}}\), \(k_{O_c}\) and \(k_{\mu_{145}}\) represent their respective innate degradation rates. \(H^{S-}(\mu_{145}, \lambda_{m_{O_c}, \mu_{145}})\) represents the inhibition of OCT4 by miR-145; \(H^{S-}(Z, \lambda_{Z,\mu_{145}})\) and \(H^{S-}(O_c, \lambda_{O_c, \mu_{145}})\) denote the inhibition of miR-145 by ZEB and OCT4 respectively.
miR-200/ZEB/OVOL circuit

There are five components in the miR-200/ZEB/OVOL module - microRNA miR-200 (\(m_{200}\)), ZEB mRNA (\(m_z\)), ZEB protein (\(Z\)), OVOL mRNA (\(m_o\)), and OVOL protein (\(O\)). All these species have an innate production and degradation rate. Transcriptional regulation is denoted by shifted Hill functions (\(H^{S+}\) for transcriptional activation and \(H^{S-}\) for transcriptional inhibition. Details of shifted Hill functions can be found in SI section 1). To capture the effects of miRNA, we consider both the degradation of mRNA by miRNAs (depicted by \(Y_m\)) and the inhibition of translation by miRNAs (depicted by \(L\)). Also, the miRNAs that bind to mRNAs can be degraded after forming a complex with mRNAs (depicted by \(Y\)). OVOL and ZEB transcriptionally inhibit each other (106), and OVOL is self-inhibitory(281). For the case of prostate cancer (OVOL inhibits both miR-200 and ZEB), the dynamics of miR-200 (\(m_{200}\)) can be described by the following equation:

\[
\frac{d\mu_{200}}{dt} = g_{m_{200}} H^{S+}(O,\lambda_{O,m_{200}})H^{S+}(Z,\lambda_{Z,m_{200}})H^{S+}(S,\lambda_{S,m_{200}}) - m_z Y_m(\mu_{200}) - k_{d_{200}} \mu_{200}
\]

where \(g_{m_{200}}\) and \(k_{d_{200}}\) are innate production and degradation rates of miR-200 respectively. \(H^{S+}(O,\lambda_{O,m_{200}})\) represents the inhibition of miR-200 by protein OVOL, \(H^{S+}(Z,\lambda_{Z,m_{200}})\) represents the inhibition of miR-200 by protein ZEB and \(H^{S+}(S,\lambda_{S,m_{200}})\) represents the inhibition of miR-200 by protein SNAIL. \(Y_m(\mu_{200})\) represents the degradation rate of miR-200 caused by forming complex with ZEB mRNAs. Regulatory links between OVOL and ZEB can be tissue-dependent. In prostate cancer, but not breast cancer, OVOL activates STAT3(282) that can inhibit miR-200(209). Therefore, for the case of breast cancer (OVOL inhibits only ZEB and not miR-200), the term \(H^{S+}(O,\lambda_{O,m_{200}})\) is omitted.

Dynamics of ZEB mRNA (\(m_z\)) and ZEB protein (\(Z\)) is described by the following equations:
where \( g_{m_z} \) and \( g_Z \) are the innate production rates of ZEB mRNA and ZEB protein respectively, and \( k_{m_z} \) and \( k_Z \) are their respective innate degradation rates. \( H^S \left( O, \lambda_{O,m_z} \right) \) denotes transcriptional inhibition of ZEB mRNA by protein OVOL, \( H^{S+} \left( Z, \lambda_{Z,m_z} \right) \) denotes the transcriptional self-activation of ZEB mRNA, and \( H^{S+} \left( S, \lambda_{S,m_z} \right) \) denotes the transcriptional activation of ZEB mRNA by protein SNAIL. \( Y_m \left( \mu_{200} \right) \) represents the degradation of ZEB mRNA by forming complex with miR-200, and \( L \left( \mu_{200} \right) \) denotes the translational inhibition of ZEB mRNA by miR-200.

Dynamics of OVOL mRNA \( (m_O) \) and protein \( (O) \) is described by the following equations:

\[
\frac{d m_O}{d t} = g_{m_O} H^S \left( O, \lambda_{O,m_O} \right) H^{S+} \left( Z, \lambda_{Z,m_O} \right) - k_{m_O} m_O.
\]

\[
\frac{d O}{d t} = g_O m_O - k_O O
\]

where \( g_{m_O} \) and \( g_O \) are the innate production rates of OVOL mRNA and OVOL protein respectively, and \( k_{m_O} \) and \( k_O \) are their respective degradation rates. \( H^S \left( O, \lambda_{O,m_O} \right) \) represents the transcriptional self-inhibition of OVOL mRNA, and \( H^S \left( Z, \lambda_{Z,m_O} \right) \) represents the transcriptional inhibition of OVOL mRNA by protein ZEB.
Parameter sensitivity analysis

Parameters used for Hill functions in various circuits are given in Tables S2.1, S2.2. Innate degradation rate for proteins ZEB, GRHL2, and OCT4 are chosen to be 0.1 hour\(^{-1}\), and those for miR-200 and miR-145 to be 0.05 hour\(^{-1}\) and 0.01 hour\(^{-1}\) respectively. Translation rate is 100 proteins per mRNA per hour for ZEB and 200 proteins per OCT4 and OVOL mRNA per hour. A detailed explanation for the estimation of these values is already given in Appendix 1. For hypothetical self-activation of GRHL2, \(n_{G, m_0} = 2\) and \(G_{m_0}^0 = 40000\) molecules. Also, for the hypothetical inhibition of miR-200 by GRHL2, as considered later, \(n_{G, m_30} = 1\) and \(G_{m_30}^0 = 80000\) molecules.

<table>
<thead>
<tr>
<th>Description</th>
<th>Fold change</th>
<th>Value</th>
<th># binding sites</th>
<th>Value</th>
<th>Threshold</th>
<th>Molecules</th>
</tr>
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<tbody>
<tr>
<td>Inhibition of ZEB by GRHL2</td>
<td>0.1</td>
<td>(n_{G, m_0})</td>
<td>1</td>
<td></td>
<td></td>
<td>25000</td>
</tr>
<tr>
<td>Inhibition of GRHL2 by ZEB</td>
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<td>(n_{Z, m_0})</td>
<td>3</td>
<td></td>
<td></td>
<td>10000</td>
</tr>
<tr>
<td>Self-activation of ZEB</td>
<td>7.5</td>
<td>(n_{Z, m_2})</td>
<td>2</td>
<td></td>
<td></td>
<td>25000</td>
</tr>
<tr>
<td>Activation of miR-200 by p53</td>
<td>5</td>
<td>(n_{P, m_200})</td>
<td>2</td>
<td></td>
<td></td>
<td>150000</td>
</tr>
<tr>
<td>Inhibition of miR-200 by ZEB</td>
<td>0.1</td>
<td>(n_{Z, m_200})</td>
<td>3</td>
<td></td>
<td></td>
<td>220000</td>
</tr>
<tr>
<td>Activation of ZEB by SNAIL</td>
<td>10</td>
<td>(n_{S, m_2})</td>
<td>2</td>
<td></td>
<td></td>
<td>180000</td>
</tr>
<tr>
<td>Inhibition of miR-200 by SNAIL</td>
<td>0.1</td>
<td>(n_{S, m_200})</td>
<td>2</td>
<td></td>
<td></td>
<td>180000</td>
</tr>
<tr>
<td>Inhibition of OCT4 by miR-145</td>
<td>0.1</td>
<td>(n_{P, m_200})</td>
<td>1</td>
<td></td>
<td></td>
<td>15000</td>
</tr>
<tr>
<td>Inhibition of OCT4 by miR-145</td>
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<td>(n_{P, m_200})</td>
<td>1</td>
<td></td>
<td></td>
<td>500000</td>
</tr>
<tr>
<td>Inhibition of ZEB by miR-145</td>
<td>0.1</td>
<td>(n_{Z, m_200})</td>
<td>1</td>
<td></td>
<td></td>
<td>100000</td>
</tr>
<tr>
<td>Inhibition of ZEB by miR-145</td>
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<td>(n_{Z, m_200})</td>
<td>1</td>
<td></td>
<td></td>
<td>1000</td>
</tr>
<tr>
<td>External signal (SA/SI) on GRHL2</td>
<td>2 (0.2)</td>
<td>(n_{S, m_30} = n_{S, m_30})</td>
<td>2</td>
<td></td>
<td></td>
<td>100000</td>
</tr>
<tr>
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<td>(n_{I, m_2})</td>
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<td></td>
<td></td>
<td>4000</td>
</tr>
<tr>
<td>Inhibition of miR-200 by I</td>
<td>0.6</td>
<td>(n_{I, m_200})</td>
<td>1</td>
<td></td>
<td></td>
<td>4000</td>
</tr>
</tbody>
</table>

Table S2.1: Parameters for miR-200/ZEB/GRHL2 and miR-200/ZEB/miR-145/OCT4 circuits
Table S2.2: Parameters used for miR-200/ZEB/OVOL

Parameter sensitivity analysis for miR-200/ZEB/GRHL circuit

To test the sensitivity of our predictions based on the parameters listed above, we conduct parameter sensitivity analysis by varying each parameter at one time. All the parameters – production rates, degradation rates, thresholds and weight factors in the shifted Hill functions are varied by +/-10%. The number of binding sites for the different interactions have been kept fixed as most of them are directly determined from experimental data. Sensitivity of the model is measured by how changes in the parameters affect the range of SNAIL levels for which the hybrid E/M phenotype exists (either alone in or combination with other phenotypes).
Figure S2.1: Parameter sensitivity analysis for the miR-200/ZEB/GRHL2 circuit (at p53=0) driven by the input signal SNAIL. Alphanumeric codes on x-axis represent the cases with different changed parameters by +/- 10%. Number 1 after the alphanumeric code represents the increase of the parameter by 10% and number 2 after the alphanumeric code represents the decrease of the parameter by 10%. Case 0 represents the result in this work, which is the control case. (A) represents the cases with +/- 10% changes in the production and degradation rates. A1 and A2 represent the
increase and decrease in the production rate of miR-200 (denoted by $g_{m_{200}}$) by 10% respectively. B1 and B2 represent the increase and decrease in the production rate of ZEB mRNA by 10% (denoted by $g_{z}$) respectively. C1 and C2 represent the increase and decrease in the production rate of ZEB protein (denoted by $g_{z}$) by 10% respectively. D1 and D2 represent respectively the increase and decrease in the production rate of GRHL2 (denoted by $g_{m_{G}}$) by 10%. E1 and E2 represent the increase and decrease in production rate of GRHL2 (denoted by $g_{G}$) by 10%. F1 and F2 represent the increase and decrease in the degradation rate of miR-200 (denoted by $k_{m_{200}}$) by 10% respectively. G1 and G2 represent the increase and decrease in the degradation rate of ZEB mRNA by 10% (denoted by $k_{z}$) respectively. H1 and H2 represent the increase and decrease in the degradation rate of ZEB protein (denoted by $k_{z}$) by 10% respectively. I1 and I2 represent respectively the increase and decrease in degradation rate of GRHL2 (denoted by $k_{m_{G}}$) by 10%. J1 and J2 represent the increase and decrease in the degradation rate of GRHL2 (denoted by $k_{G}$) by 10%. (B) represents the cases with changes in the thresholds in the shifted Hill functions. K1 and K2 represent respectively the increase and decrease in the threshold levels for the inhibition of miR-200 by ZEB (denoted by $Z_{0}^{0}$) by 10%. L1 and L2 represent the 10% increase and decrease in the threshold levels of SNAIL inhibition on miR-200 (denoted by $S_{0}^{0}$) respectively. M1 and M2 represent the increase and decrease in the threshold levels of SNAIL activation on ZEB (denoted by $S_{m_{Z}}^{0}$) by 10%. N1 and N2 represent the increase and decrease in the threshold levels of miR-200 (denoted by $Z_{m_{Z}}^{0}$) by 10%. O1 and O2 represent the case for 10% increase and decrease in threshold levels of ZEB for its self-activation (denoted by $Z_{m_{Z}}^{0}$). P1 and P2 represent 10% increase and decrease in threshold levels of GRHL2 for inhibition of ZEB (denoted by $G_{m_{G}}^{0}$) and Q1 and Q2 denote 10% increase and decrease in threshold levels of ZEB for inhibiting GRHL2 (denoted by $Z_{m_{G}}^{0}$). (C) represents the cases with changes in the weight factors in the shifted Hill functions. R1 and R2 denote the increase and decrease in the weight factor of ZEB inhibition on miR-200 (denoted by $z_{m_{200}}$) by 10%. S1 and S2 represent the respective increase and decrease in the weight factor of SNAIL inhibition on miR-200 (denoted by $s_{m_{200}}$) by 10%. T1 and T2 denote respective increase and decrease in weight factor of SNAIL activation on ZEB (denoted by $s_{m_{Z}}$). U1 and U2 denote the respective increase and decrease in weight factor of GRHL2 inhibition of ZEB (denoted by $\lambda_{m_{Z}}^{G}$) by 10%. T1 and T2 denote the respective increase and decrease in weight factor of inhibition of GRHL2 by ZEB (denoted by $\lambda_{m_{G}}^{Z}$) by 10%. U1 and U2 represent the respective increase and decrease in the weight factor of ZEB self-activation (denoted by $z_{m_{Z}}^{Z}$) by 10%. Dotted boxes represent cases when the range of SNAIL levels
for the existence of hybrid E/M phenotype is affected largely – either decreased or increased.

Our previous analysis shows that the model for (miR-200/ZEB) circuit is sensitive towards low values of miR-200, i.e. changes in the parameters which decrease the levels of miR-200 decrease the overall range of SNAIL levels for the existence of the E/M phenotype (186). Coupling GRHL2 with miR-200/ZEB enlarges the range of SNAIL levels for the existence of a hybrid E/M region (Figure 3.2A, B). We observe that when parameters are increased or decreased by 10%, the absolute levels of SNAIL for which the hybrid E/M phenotype exists changes in most cases (Figure S2.1). However, here we focus on the change in the range of SNAIL levels for the existence of the hybrid E/M state, when different parameters are varied. Compared with the control case (no parameter is changed – case 0 in Figure S2.1A-C), the range of SNAIL levels for the existence of an E/M state decreases significantly when the production rate for ZEB mRNA is increased (case B1), the degradation rate for ZEB mRNA is decreased (case G2) (Figure S2.1A), threshold of ZEB levels for the shifted Hill function of inhibition of ZEB on miR-200 is decreased (case K2) (Figure S2.1B), and the strength of ZEB activation by SNAIL is increased (case T1), and the strength of ZEB self-activation is increased (case W1) (Figure S2.1C). All these cases represent cases where the effective ZEB levels that can inhibit miR-200 and drive EMT are increased, i.e. the propensity of the cell to undergo EMT is increased. Also, consistently, at increased ZEB levels, GRHL2 levels are decreased (ZEB inhibits GRHL2) and hence the effect of GRHL2 in expanding the hybrid E/M range is decreased.

Conversely, the parameter changes that are likely to decrease the effective ZEB levels that can induce EMT should enlarge the range of SNAIL levels for which the hybrid E/M state exists. This is indeed observed for cases such as when the production rates of ZEB mRNA and ZEB protein are decreased (cases B2, C2), the threshold of ZEB levels for the shifted Hill function of inhibition of ZEB on miR-200 is increased (case K1), and the strength of ZEB activation by SNAIL is decreased (case T2), and the strength of ZEB self-activation is decreased (case W2) (Figure S2.1). Thus, a change in several parameters of the miR-200/ZEB/GRHL2 circuit, especially those affecting the
ZEB protein levels, can affect the range of SNAIL levels for which the hybrid E/M state exists. The change of parameters with respect to GRHL2 did not affect this range.

Parameter sensitivity analysis for miR-200/ZEB/miR-145/OCT4 circuit

Similar to the sensitivity analysis for miR-200/ZEB/GRHL2 circuit, we observe that for a 10% increase or decrease in the parameters, the absolute levels of SNAIL for which the hybrid E/M phenotype exists changes mildly in most cases (Figure S2.2). However, focusing on the change in the range of SNAIL levels for the existence of hybrid E/M state, we find that compared with the control case (no parameter changed – case 0 in Figure S2.2A-C), the range of SNAIL for the existence of E/M state decreases relatively significantly when the production rate of ZEB mRNA is increased (case B1) or its degradation rate is decreased (case H2) (Figure S2.2A), threshold of ZEB levels for the shifted Hill function of inhibition of ZEB on miR-200 is decreased (case M2) (Figure S2.2B), and the strength of ZEB activation by SNAIL is increased (case Y1), strength of ZEB self-activation is increased (case AB1), and the strength of miR-145 inhibiting ZEB is weakened (case AC1) (Figure S2.2C). All these cases represent cases where the effective ZEB levels that can inhibit miR-200 and drive EMT are increased, i.e. the propensity of the cell to undergo EMT is increased. Also, consistently, at increased ZEB levels, miR-145 levels are decreased (ZEB inhibits miR-145) and hence the effect of miR-145 in expanding the hybrid E/M range is decreased.

Conversely, the parameter changes that are likely to decrease the effective ZEB levels that can induce EMT should enlarge the range of SNAIL levels for which the hybrid E/M state exists. This is indeed observed for cases such as when the production rate of ZEB mRNA is decreased (case B2), the degradation rate of ZEB mRNA is increase (case H1), the threshold ZEB levels for shifted Hill function of inhibition of ZEB on miR-200 is increased (case M1), the strength of ZEB activation by SNAIL is decreased (case Y2), the strength of ZEB self-activation is decreased (case AB2), and strength of inhibition of ZEB by miR-145 is increased (case AC2) (Figure S2). Therefore, for most parameter changes (either increase or decrease), the region of SNAIL levels for which the hybrid E/M exists does not change much, as long as the ZEB levels are not very high, thereby
suggesting that our prediction regarding the ‘phenotypic stability factor’ role of GRHL2 and miR-145/OCT4 is quite robust to parameter variation. Similar results were obtained for OVOL1/2 both for the cases of breast and prostate cancer(99).

A

Cases of changes in production/degradation rates for miR-200/ZEB/miR-145/OCT4

B

Cases of changes in Hill function thresholds for miR-200/ZEB/miR-145/OCT4

C

Cases of changes in weight factors for miR-200/ZEB/miR-145/OCT4
Figure S2.2: Parameter sensitivity analysis for the miR-200/ZEB/miR-145/OCT4 circuit driven by the input signal SNAIL. Alphanumeric codes on x-axis represent the cases with different changed parameters by +/- 10%. Number 1 after the alphanumeric code represents the increase of the parameter by 10% and number 2 after the alphanumeric code represents the 10% decrease in parameter. Case 0 represents the result in this work, which is the control case. (A) represents the cases with +/- 10% changes in the production and degradation rates. A1 and A2 represent the increase and decrease in the production rate of miR-200 (denoted by $g_{\mu_{200}}$) by 10% respectively. B1 and B2 represent the increase and decrease in production rate of ZEB mRNA (denoted by $Z_m$) by 10%. C1 and C2 represent the increase and decrease in production rate of ZEB protein (denoted by $Z_g$) by 10%. D1 and D2 represent increase and decrease in the production rate of miR-145 (denoted by $g_{\mu_{45}}$) by 10%. E1 and E2 represent increase and decrease in production rate of OCT4 mRNA (denoted by $g_{\mu_{Oc}}$) by 10%. F1 and F2 represent increase and decrease in the production rate of OCT4 protein (denoted by $g_{cO}$) by 10%. G1 and G2 represent increase and decrease in the degradation rate of miR-200 by 10% (denoted by $k_{\mu_{200}}$). H1 and H2 represent increase and decrease in degradation rate of ZEB mRNA by 10% (denoted by $k_{Z_m}$). I1 and I2 represent increase and decrease in degradation rate of ZEB protein by 10% (denoted by $k_{Z_g}$). J1 and J2 represent the increase and decrease in degradation rate of miR-145 (denoted by $k_{\mu_{45}}$) by 10%. K1 and K2 represent the increase and decrease in degradation rate of OCT4 mRNA by 10% (denoted by $k_{m_{Oc}}$). L1 and L2 represent increase and decrease in degradation rate of OCT4 protein by 10% (denoted by $k_{cO}$). (B) represents the cases with changes in the thresholds in the shifted Hill functions. M1 and M2 represent increase and decrease in the threshold levels for the inhibition of miR-200 by ZEB (denoted by $Z_{200}$) by 10%. N1 and N2 represent the case for 10% increase and decrease in threshold levels of ZEB for self-activation (denoted by $Z_{mZ}$). O1 and O2 represent the 10% increase and decrease in the threshold levels of SNAIL inhibition on miR-200 (denoted by $S_{200}$) respectively. P1 and P2 represent the increase and decrease in the threshold levels of SNAIL activation on ZEB (denoted by $S_{mZ}$) by 10%. Q1 and Q2 represent the increase and decrease in the threshold levels of miR-200 (denoted by $S_{200}$) by 10%. R1 and R2 represent 10% increase and decrease in threshold levels of OCT4 for inhibiting miR-145 (denoted by $O_{c45}$). S1 and S2 denote 10% increase and decrease in threshold levels of OCT4 for activating miR-200 (denoted by $O_{\mu_{200}}$). T1 and T2 denote 10% increase and decrease in threshold levels of miR-145 for inhibiting OCT4 (denoted by $\mu_{145Oc}$). U1 and U2 denote 10% increase and decrease in threshold levels of miR-145 for inhibiting ZEB (denoted by $\mu_{145Z_m}$). V1 and V2 denote 10% increase and decrease in threshold levels of ZEB for
inhibiting miR-145 (denoted by $Z_{\text{m}145}$). (C) represents the cases with changes in the weight factors in the shifted Hill functions. W1 and W2 denote increase and decrease in weight factor of ZEB inhibition on miR-200 (denoted by $Z_{\text{m}200}$) by 10%. X1 and X2 represent increase and decrease in weight factor of SNAIL inhibition on miR-200 (denoted by $S_{\text{m}200}$) by 10%. Y1 and Y2 denote respective increase and decrease in weight factor of SNAIL activation on ZEB (denoted by $s_{\text{m}Z}$). Z1 and Z2 denote the respective increase and decrease in weight factor of OCT4 inhibition of miR-145 (denoted by $\lambda_{\text{m}145}$) by 10%. AA1 and AA2 denote increase and decrease in weight factor of inhibition of OCT4 by miR-145 (denoted by $\lambda_{\text{m}145}$) by 10%. AB1 and AB2 denote increase and decrease in weight factor of ZEB self-activation (denoted by $Z_{\text{m}Z}$) by 10%. AC1 and AC2 denote increase and decrease in weight factor of miR-145 inhibiting ZEB (denoted by $\lambda_{\text{m}145}$) by 10%. AD1 and AD2 denote increase and decrease in weight factor of ZEB inhibiting miR-145 (denoted by $\lambda_{\text{m}Z}$) by 10%. AE1 and AE2 denote increase and decrease in the weight factor of activation of miR-200 by OCT4 (denoted by $\lambda_{\text{m}200}$) by 10%.

Levels of CDH1, VIM, ZEB, and PSFs in H2291, H1975, and H1299 cells

![Image A: H2291 DAPI, VIM, CDH1, Merged]

![Image B: Bar graph showing relative abundance of CDH1, VIM, GRHL2, OVCL2, ZEB1, SNAI1 in H1975 and H1299]

![Image C: Western blots for CDH1, VIM, GAPDH in H1975 and H1299]
Figure S2.3: Levels of canonical epithelial and mesenchymal genes, EMT-inducing players and PSFs (A) Expression of CDH1 (E-cadherin) and VIM (Vimentin) in H2291 cells. Scale bar 100μm. (B) RT-PCR analysis of CDH1, VIM, OVOL2, GRHL2, ZEB1, and SNAI1 in H1975 (blue) and H1299 (orange). (C) Western-blot of H1975 and H1299.

Figure S2.4: Expression of CDH1 (E-cadherin) and VIM (Vimentin) in different passages of the H1975 cells, as examined by immunofluorescence staining. Scale bar 100μm. P7 (passage #7) and P12 (passage #12) are separated by a period of two months.

OVOL1/2 can behave as a PSF

The response of miR-200/ZEB/OVOL circuit to SNAIL as an external signal, both for the case of prostate cancer and breast cancer, is presented in Figure S2.5 through bifurcation diagram and phase diagrams where SNAIL is considered as two separate external signals – one activating ZEB and the other inhibiting miR-200. Similarly, investigating the dynamics of the miR-200/ZEB/OVOL circuit indicates that EMT and
MET are both two-step processes, and that in presence of OVOL, cells can maintain a hybrid E/M phenotype more stably (compare brown rectangles in Figure S2.6B,C).

**Figure S2.5: Dynamical system characteristics of the miR-200/ZEB/OVOL circuit.** A. Bifurcation of mRNA levels of ZEB in response to SNAIL levels for miR-200/ZEB circuit. It illustrates the possible co-existence (for some range of SNAIL levels) of the three possible stable states for same physiological conditions - E - (1, 0), E/M - (½, ½) and M - (0,1). B. Bifurcation of mRNA levels of ZEB in response to SNAIL levels for miR-200/ZEB/OVOL circuit (without the inhibition of miR-200 by OVOL). C. Bifurcation of mRNA levels of ZEB in response to SNAIL levels for miR-200/ZEB/OVOL circuit (with the inhibition of miR-200 by OVOL). Phase-diagram driven by two independent signals S1 and S2 representing SNAIL, as illustrated in the Inset circuit for D. miR-200/ZEB circuit, E. miR-200/ ZEB/OVOL circuit (without the inhibition of miR-200 by OVOL), and F. miR-200/ZEB/OVOL circuit (with the
inhibition of miR-200 by OVOL). Each phase (denoted by a different color) corresponds to a different combination of co-existing states or phenotypes. The bifurcation diagram and phase diagram in every row are for the circuit drawn in the leftmost column of that row. The region marked by purple dots in B, C represents the range of SNAIL levels for which the hybrid E/M phenotype can exist alone, and the region marked by green in (A), (B), (C), and that by black dots in (D), (E), (F) represents the range of SNAIL levels for which the hybrid E/M phenotype can exist alone or as one of the multiple possible phenotypes.
**Figure S2.6: Temporal dynamics of epithelial-hybrid-mesenchymal transitions for miR-200/ZEB/OVOL circuit.** A. Time-varying external signal (SNAIL levels) applied to miR-200/ZEB/OVOL circuit (with the inhibition of miR-200 by OVOL). B. Temporal evolution of miR-200 (green, scaled by 0.02 to fit in the plot) and ZEB mRNA (blue) for the miR-200/ZEB module. This figure shows that EMT is a two-step process, E->E/M->M, while MET is a one-step process, from M->E directly. C. Temporal evolution of miR-200 (green, scaled by 0.02 to fit in the plot), ZEB mRNA (blue) and protein OVOL (purple, scaled by 0.02 to fit in the plot) for the miR-200/ZEB/OVOL module. Areas shown in the boxes (days 5-12 and days 25-32) are expanded in D. and E. to show that the cells pass through the hybrid E/M state while undergoing EMT or MET. Different colors in B–E represent different stable states or phenotypes - cyan for E or (1,0) state, brown for hybrid E/M or (½, ½) state, yellow for M or (0,1) state.

**Effect of external signal on miR-200/ZEB/GRHL2 circuit**

An external activation signal on GRHL2 (SA) largely increases the area of the region corresponding to the epithelial phenotype - \{E\} and \{E, E/M\}; while an inhibition signal (SI) reduces this area and increases the area of the region corresponding to mesenchymal phenotype – \{E, E/M, M\} and \{E/M, M\} and \{M\} (Figure S2.7A, B), suggesting that GRHL2 knockdown can lead to a complete EMT, and that its overexpression can lead to MET. Similar results have been obtained for OVOL2 in our earlier theoretical analysis (99). Further, activating miR-200 by p53 can drive MET (as reported (283)) both in presence and absence of GRHL2 (Figure S2.7C) that can behave as a ‘phenotypic stability factor’ for different values of p53 (Figure S2.7D).

**Knockdown of GRHL2 and OVOL2**

Knockdown of GRHL2 and OVOL2 led to an increase in ZEB1 levels approximately 4-fold (Figure S2.8A) however, corresponding increase in SNAI1 was relatively less (Figure S2.8B), potentially because both GRHL2 and OVOL2 directly target ZEB1 and not SNAI1. Also, knockdown of GRHL2 tends to impact the proliferation for H1975 cells as they tend to undergo a complete EMT now. Unless otherwise specified, the figures (Figure 3.3, S2.8C, D) corresponding to GRHL2 and OVOL2 knockdown show the results for siGRHL#2 and siOVOL2#2.
Figure S2.7: Phase diagram for miR-200/ZEB/GRHL2 circuit in response to SNAIL, external signals on GRHL2, and p53. (A) Response of the circuit to variable levels of SNAIL and an external activation signal on GRHL2 (SA). (B) shows the same as (A), but for an external inhibition signal on GRHL2 (SI). Different colors represent different phases (co-existence of possible stable states). (C) Response of the miR-200/ZEB circuit to variable levels of SNAIL and p53. (D) shows the same as (C), but for miR-200/ZEB/GRHL2 circuit. The area bounded by dotted black lines denotes the range of parameters for which the hybrid E/M state exists, either alone or in combination with other phenotypes. Different colored areas show different phases (co-existing phenotypes).
Figure S2.8: Characterizing the knockdown of GRHL2 and OVOL2 in H1975 cells. (A, B) RT-PCR analysis of ZEB1 and SNAI1 levels upon knockdown of GRHL2 and OVOL2. N=3 for each bar. (C) Cell viability at 72 and 96 hours after treatment with siGRHL2 and siOVOL2. (D) Comparing the area covered during scratch assay of H1975, H1975-siOVOL2, H1975-siGRHL2 cells. *: p<0.05

Analysis of NCI 60 and PC3 clonal cell lines

Fold-change in levels of ZEB1, CDH1 and CDH3 suggest that OVOL2 might be a stronger driver of MET than OVOL1 in PC3 cells. Care must be exercised in interpreting the values for OVOL1 in PC3-EMT-OVOL1 and that for OVOL2 in PC3-EMT-OVOL2 (marked by red in Figure S2.9A), because of the use of an overexpression vector. Nevertheless, OVOL2 being a strong driver was also reported in mammary development (35), and potentially explains why OVOL2-KO as compared to OVOL1-KO in H1975 cells is expected to yield a stronger phenotype. ZEB1, OVOL2, CDH3, and GRHL2 have statistically significant different levels across the three phenotypes – E, M, and E/M – with the only exception being GRHL2 levels in E/M and M sets (Figure S2.9B). This lack of significance can be attributed to tissue-specific differences as well as the presence
of a few cell lines that have low (instead of medium) expression of CDH1 and VIM in hybrid E/M cell line cluster (118).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Epi/EMT</th>
<th>EMT-OVOL2/EMT</th>
<th>EMT-OVOL1/EMT</th>
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<tr>
<td>ZEB1</td>
<td>0.02</td>
<td>0.24</td>
<td>0.57</td>
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<td>CDH1</td>
<td>50.43</td>
<td>40.41</td>
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<td>142.12</td>
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<td>17.79</td>
<td>8.94</td>
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<tr>
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<td>25.89</td>
<td>2243.44</td>
<td>1.50</td>
</tr>
<tr>
<td>OVOL1</td>
<td>174.43</td>
<td>2.97</td>
<td>473.47</td>
</tr>
</tbody>
</table>

**Figure S2.9:** Analysis of NCI-60 panel and PC3-EMT-OVOL1, PC3-EMT-OVOL2. (A) Fold-change in the mean expression (log2) of OVOL2, OVOL1, ZEB1, CDH1, CDH3, GRHL2 in PC3-EMT-OVOL1 or 2, and PC3-Epi vs. PC3-EMT fold changes (B) Mean expression (log 2) of GRHL2, OVOL2, CDH3, and ZEB1 in different cell lines categorized as E, M and hybrid E/M. *, p<=0.05; **, p<0.005; ***, p<0.0005. Error bars represent standard error of mean.

**GRHL2, OVOL2, and CDH3 levels may predict poor survival**
Figure S2.10: Survival analysis. Overall survival, relapse-free survival and metastasis-free survival for the expression of GRHL2, OVOL2 and CDH3 individually in multiple tissue types – (A) GSE14333 (n=187), (B) GSE17536 (n=135), (C) GSE31210 (n=225), (D) GSE41271 (n=274), (E) GSE48408 (n=163), (F) GSE24551 (n=159), (G) NKI (n=294), (H) GSE 6532_U133A (n=178), (I) GSE42568 (n=103).

We observed that in datasets where any one of the players – GRHL2, OVOL2, and CDH3 – correlates with poor survival, a combination of more than one of them also correlates with poor survival and usually has a lower p-value than the case with only one player. Also, we observed at least one case (GSE 3494 – n=235 patients) where the combined expression of GRHL2, OVOL2, and CDH3 correlated with poor overall survival in a statistical significant way, but none of them individually did (Table S2.4), indicating that looking at the combined expression might yield more predictive power. Looking at the combined expression might also help mitigate tissue-specific differences in expression levels of these players, each of which has been predicted to specifically associate with a hybrid E/M phenotype.

<table>
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<th>Predictor variables</th>
<th>Hazard ratio</th>
<th>p-value</th>
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<tr>
<td>GRHL2</td>
<td>1.25 (0.67-2.33)</td>
<td>0.4921</td>
</tr>
<tr>
<td>OVOL2</td>
<td>1.15 (0.58-2.29)</td>
<td>0.6942</td>
</tr>
<tr>
<td>CDH3</td>
<td>1.25 (0.96-1.62)</td>
<td>0.0935</td>
</tr>
<tr>
<td>GRHL2, OVOL2</td>
<td>1.4 (0.57-3.46)</td>
<td>0.4618</td>
</tr>
<tr>
<td>GRHL2, CDH3</td>
<td>1.51 (0.95-2.39)</td>
<td>0.0801</td>
</tr>
<tr>
<td>OVOL2, CDH3</td>
<td>1.77 (1-3.12)</td>
<td>0.0497</td>
</tr>
<tr>
<td>GRHL2, OVOL2, CDH3</td>
<td>2.11 (1-4.46)</td>
<td>0.0497</td>
</tr>
</tbody>
</table>

Table S2.4: Correlation of GRHL2, OVOL2, and CDH3 either individually or in combination with overall survival for GSE 3494.

Network motifs to identify further PSFs
Figure S2.11: Reduced or effective miR-200/ZEB/miR-145 circuit, where miR-145 both inhibits miR-200 and indirectly self-activates via OCT4.

Mutual inhibition between OCT4 and miR-145 can be considered to a self-activation due to the action of miR-145, i.e. inhibiting its own inhibitor. Furthermore, the inhibition of miR-200 by miR-145 via OCT4 can therefore be replaced with a direct inhibitory link. The inhibition of miR-145 on ZEB is expected to be relatively stronger because in addition to directly inhibiting ZEB, miR-145 can also inhibit the nuclear translocation of β-catenin that can fuel the completion of ZEB by activating the TCF4 complex that can further activate ZEB and lead to more release of membranous β-catenin. Nonetheless, this reduction into the effective circuit is likely to depend on relative strengths of the links in the network (Figure S2.11).

Figure S2.12: Bifurcations for coupling of miR-200/ZEB in different topologies. (A) An external incoherent signal that inhibits both miR-200 and ZEB. (B) An external incoherent signal that activates both miR-200 and ZEB. (C) A double
negative feedback loop with miR-200. Bifurcation diagrams below correspond to the behavior of the circuits just above. The green shaded region represents the range of SNAIL levels for which the hybrid E/M phenotype exists either alone or in combination with other phenotypes. The dotted rectangle denotes the range of SNAIL levels for which hybrid the E/M phenotype can exist alone. Blue solid lines represent stable steady states (phenotypes), and red dashed lines show unstable steady states.

The proposed motif (iii) is also supported by the analysis done for miR-200/ZEB/OVOL circuit for the case of prostate cancer where the inhibition of OVOL by ZEB is varied. We calculate a phase diagram for SNAIL and $\lambda_{Z,\text{m}}$ (fold-change in transcription rate of OVOL due to repression by ZEB), when the prostate cancer circuit is driven by an external inhibition signal (SI) on OVOL (Figure S2.13A). We found that the {E/M} phase exists only when ZEB inhibits OVOL weakly (Figure S2.13B). Further, in phase diagrams of SNAIL and SI as the external signals, the {E/M} phase was seen only for a weak inhibition of OVOL by ZEB (Figure S2.13C, D), thus consistent with our results that OVOL acts as an ‘expander’ of the E/M phenotype, and our insights from sensitivity analysis of the model for miR-200/ ZEB/OVOL circuit both for prostate and breast cancer.

Materials and methods

Cell line and siRNA transfection
H1975 cell line was authenticated and free from mycoplasma, grown in RPMI 1640 with 10% FBS and 1% penicillin/streptomycin. siRNA against GRHL2, OVOL2 and scrambled control siRNA were purchased from Sigma (Hs01_00105962, Hs01_00105964, Hs02_00357526, Hs01_00357580). siRNA transfection was performed with Lipofectamine 2000 (# 11668-019; Invitrogen), according to the manufacturer’s instructions. siRNA concentration of 50 nmol was used for a 35 mm dish of H1975 cells.

Wound-healing assay
A scratch or wound-healing assay was performed to determine cell migration using confluent cultures (80%–90% confluence). Briefly, cells (1 × 105 cells/ml) were seeded in 6-well tissue culture plate and grown to confluence. Cells were starved for 24 hours using 0.2% serum in growth media and treated with mitomycin to minimize cell proliferation. The next day, the confluent monolayer was scratched with a sterile p200 pipet tip and media replaced with complete growth media. Images were acquired at 0 and 12 hours; the assay was performed at least twice per cell line. The quantification of area covered was done by ImageJ software.
Figure S2.13: Phase-diagrams of miR-200/ZEB/OVOL circuit for prostate cancer with different strengths of inhibition of OVOL by ZEB. A) miR-200/ZEB/OVOL circuit for prostate cancer. $\lambda_{Z,m_0}$ is the fold-change from basal synthesis rate of OVOL mRNA by binding of protein ZEB. Since ZEB transcriptionally inhibits OVOL, $\lambda_{Z,m_0} < 1$. The smaller the $\lambda_{Z,m_0}$, the stronger the inhibition of ZEB on OVOL. B) Phase diagram of miR-200/ZEB/OVOL circuit driven by two parameters - $\lambda_{Z,m_0}$ and SNAIL levels. C) Phase diagram of miR-200/ZEB/OVOL circuit with $\lambda_{Z,m_0} = 0.1$ driven by variable levels of both SNAIL and the external inhibition signal (SI) on OVOL. D) Phase diagram of miR-200/ZEB/OVOL circuit with $\lambda_{Z,m_0} = 0.9$ driven by variable levels of both SNAIL and the external inhibition signal (SI) on OVOL. Different colors in A - D represent different phases (set of co-existing phenotypes for the same physiological conditions). Area bound by the black dots shows the total range of physiological parameters for which the hybrid E/M phenotype exists, either alone or in combination with other possible phenotypes.
Model simulation
The bifurcation plots were evaluated using MATCONT (284).

Kaplan-Meier analysis
Kaplan-Meier plots were generated using the online tool ProgGene (285). The patients were classified into high or low based on the median level of expression for a given gene.

RT-PCR analysis and immunofluorescence
Complementary DNA samples were prepared using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies). A TaqMan PCR assay was performed with a 7500 Fast Real-Time PCR System using TaqMan PCR master mix, commercially available primers, FAM™-labeled probes for GRHL2, OVOL2, CDH1, Vimentin and VIC™-labeled probes for 18S, according to the manufacturer’s instructions (Life Technologies). Each sample was run in triplicate. Ct values for each gene were calculated and normalized to Ct values for 18S (ΔCt). The ΔΔCt values were then calculated by normalization to the ΔCt value for control. For immunofluorescence, cells were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, then stained with anti-CDH1 (1:100; Abcam) and antivimentin (1:100; Cell Signaling Technology). The primary antibodies were then detected with Alexa conjugated secondary antibodies (Life technologies). Nuclei were visualized by co-staining with DAPI.
Appendix 3: Enhanced tumor-initiation potential of the hybrid E/M phenotype

**Regulatory circuit construction**

Figure 4.1A shows mutual inhibition between RNA binding factor LIN28 and microRNA let-7 – let-7 represses the translation of LIN28, while LIN28 inhibits the processing to mature let-7 by blocking the processing of let-7 precursors by Dicer in the cytoplasm by recruiting a TUTase (Zcchc11/TUT4), and by inhibiting the processing of primary let-7 transcripts by the Micro-processor Drosha (195, 286). Also, they both self-activate – let-7 promotes its own processing (287), and LIN28 promotes its own translation (207), thereby forming a self-activating toggle switch (96). Furthermore, the oncogene c-MYC transcriptionally activates LIN28 by binding to three conserved E-box sequences in the transcriptional start site (TSS) of LIN28 (203), but inhibits let-7, both through LIN28 (203) as well as by directly binding to two E-boxes in its TSS (204). NF-kB transcriptionally activates both LIN28 through multiple conserved NF-kB motifs (288); also, it weakly activates let-7 transcriptionally (206). LIN28 activates the translation of OCT4 (197), that induces the transcriptional activation of miR-200 (198). miR-200 inhibits the translation of LIN28 through multiple consensus sequences (196). Table S3.1 below characterizes each interaction in the circuit shown in Figure 1A. However, as mentioned in the legend for Figure 4.1B, we have not included the activation of miR-200 by OCT4 in our current analysis.
RNA binding factor LIN28 inhibits the biogenesis of microRNA let-7 by halting its processing (195, 286). This is a novel model of regulation as compared to canonical transcriptional (by transcription factors), translational (by microRNAs) or post-translational (by kinases etc.) modes. LIN28 binds to terminal loop (TL) of pre-let-7 cooperatively, thereby enabling multiple LIN28 molecules to bind to the TL, and preventing the processing of pre-let-7 by Dicer (289). Experiments identify this binding effect to be a Hill function (289), therefore we use a shifted Hill function to consider the effect of LIN28 on let-7 processing (Equation (1)). Shifted Hill functions

\[ H^s(X, \lambda) = H^-(X) + \lambda H^+(X) \] (97)

are defined as the weighted sum of excitatory Hill function \( H^+(X) \) and inhibitory Hill function \( H^-(X) \), where \( \lambda \) denotes the fold change in production rate of the species due to \( X \). Therefore, shifted Hill function obviates the need for having an extra term in the equation for basal rates, as it accounts for both the
basal rate of production (in absence of any regulator X) as well as the modified (higher or lower) rate of production due to the effect of X.

MicroRNA let-7 binds to its own primary transcript and promotes its processing through Argonaute (287), hence directly self-activating itself. Besides, let-7 mediates indirect self-activation by repressing its inhibitor c-MYC (204, 290). Self-activation is usually reflected by Hill function (131), hence we use shifted Hill function to represent the effect of let-7 on its processing (Equation (1)). Similarly, LIN28 promotes its own translation by binding to seven consensus sequences on its own mRNA, as shown by PAR-CLIP data (207); and we have used shifted Hill function that of LIN28 on its production (Equation (2)). Also, we have previously shown that microRNA-mediated silencing that incorporates both active mRNA degradation and translational inhibition can be approximated as a Hill function (97). Therefore, terms representing the effect of let-7 and miR-200 on LIN28 have been represented as Hill functions (Equations (S1)-(S2)).

The effect of miR-200 on the Lin28/let-7 circuit is represented by equations (S1)-(S2) given by:

\[
\frac{d\mu}{dt} = g_{\mu}H^+(B, \lambda_{B,\mu})H^+(\mu, \hat{\lambda}_{\mu,\mu}) - k_{\mu}\mu...........................(S1)
\]

\[
\frac{dB}{dt} = g_{B}mH^+(\mu, \hat{\lambda}_{\mu,B})H^+(B, \hat{\lambda}_{B,B})H^-(S) - k_{B}B...........(S2)
\]

\(H^-(S)\) denotes the inhibitory Hill function denoting miR-200 mediated translational inhibition.

Values of most of the parameters considered in our model are estimated from experimental data as explained in detail in Appendix 1. Innate degradation rates for proteins, mRNAs and miRNAs were selected according to their half-lives from experimental data as - 0.1 hour\(^{-1}\) for LIN28 (depicted by \(k_{\nu}\)), 0.5 hour\(^{-1}\) for LIN28 mRNA (depicted by \(k_{m}\)), and 0.05 hour\(^{-1}\) for let-7 (depicted by \(k_{\mu}\)). The translational rate for LIN28 (\(g_{B}\)) is estimated to be \(0.2*10^3\) proteins per mRNA per hour. Also, the values of \(\lambda\) have been set to be about ten-fold, i.e. \(\lambda\) for activation is up to 11, and \(\lambda\) for repression is
0.1. Levels of OCT4 (on a scale of 0-1) were calculated as excitatory Hill function of LIN28 levels with Hill coefficient = 2 and Hill threshold=430*10^3 molecules.

The effect of c-MYC on the LIN28/let-7 circuit is represented by equations (S3)-(S4) given by:

\[
\frac{d\mu}{dt} = g_{\mu}H^+(B, \lambda_{\mu,B})H^+(\mu, \lambda_{\mu,\mu})H^-(C_{\mu}) - k_{\mu}\mu..........................(S3)
\]

\[
\frac{dB}{dt} = g_{B}mH^+(\mu, \lambda_{\mu,B})H^+(B, \lambda_{B,B})H^-(S)(1 + 2H^+(C_{B})) - k_{B}B...........(S4)
\]

<table>
<thead>
<tr>
<th>Description</th>
<th>Parameter</th>
<th>Value</th>
<th>Parameter</th>
<th>Value</th>
<th>Threshold</th>
<th>#Molecules</th>
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<tr>
<td>Self-activation of let-7 ((\mu))</td>
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<td>(\lambda_{\mu,\mu})</td>
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<td>(\lambda_{C,B})</td>
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<td>(N_{\mu}^0)</td>
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<td>Activation of LIN28 (B) by NF-kB (N)</td>
<td>(n_{N,B})</td>
<td>2</td>
<td>(\lambda_{N,B})</td>
<td>3</td>
<td>(N_{B}^0)</td>
<td>40*10^3</td>
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<tr>
<td>Activation of OCT4 (O) by LIN28 (B)</td>
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<td>Considered as a (+ve) Hill function</td>
<td></td>
<td>(B_{O}^0)</td>
<td>430*10^3</td>
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</table>

Table S3.2: List of parameters used in the simulations of LIN28/let-7 circuit driven by miR-200, c-MYC and NF-kB. Notation used: \(\lambda_{x,y}, n_{x,y}\) denote the weight
factor and Hill coefficient for the regulation of $y$ by $x$. $x_y^0$ represent the Hill function threshold of regulation of $y$ by $x$.

Effect of c-MYC (C) on let-7 ($\mu$) is given by $H^-(C_{\mu})$, and that on LIN28 (B) by $(1+2H^+(C_{B}))$. Since the excitatory Hill function $H^+(X)$ and inhibitory Hill function $H^-(X)$ sum up to 1, $(1+2H^+(C_{B}))$ represents a shifted Hill function with $\lambda_{C,B} = 3$ [because $1+2H^+(C_{B}) = H^-(C_{B})+3H^+(C_{B})$].

Effect of NF-kB on the LIN28/let-7 circuit is represented by equations below:

$$\frac{d\mu}{dt} = g_{\mu}H^+(B, \lambda_{B,\mu})H^+(\mu, \lambda_{\mu,\mu})(1+2H^+(N_{\mu})) - k_{\mu}\mu$$ \hspace{1cm} (S5)

$$\frac{dB}{dt} = g_{B}mH^+(\mu, \lambda_{\mu,B})H^+(B, \lambda_{B,B})H^-(S)(1+2H^+(N_{B})) - k_{B}B$$ \hspace{1cm} (S6)

Effect of NF-kB on let-7 ($\mu$) is given by $(1+2H^+(N_{\mu}))$, and that on LIN28 (B) by $(1+2H^+(N_{B}))$. As explained above, both these terms are shifted Hill function with $\lambda_{N,\mu} = \lambda_{N,B} = 3$.

**Sensitivity analysis**

To understand how sensitive our predictions are to the choice of parameters listed above, we perform a sensitivity analysis by varying our parameters by ± 20% of their original values. The no. of binding sites of one species on the other (depicted by the Hill coefficient in Hill functions) has been considered to be fixed. Other parameters – degradation and production rates, thresholds (half-maximal concentrations), and weight factors of Hill functions – have been varied by ± 20%.

We first analyze the LIN28/let-7 circuit driven by miR-200 as the external signal, and find it to be tristable, i.e. LIN28/let-7 has an additional state – D/U or (medium LIN28, medium let-7) – in addition to U or (high LIN28, low let-7) and D or (low LIN28, high let-7) states (Figure 2A). In our sensitivity analysis, we have increased or decreased
each parameter by 20% (the individual cases are denoted by an alphanumerical code) and plotted the range of miR-200 levels for which D/U state exists (Figure S3.1A, B). The absolute levels of miR-200 for which D/U state exist increase or decrease for most parameters change. But, here our main focus is to identify the cases of parameter change for which the range of miR-200 levels (and not the absolute levels of miR-200) for the existence of the D/U state decreases the most. Compared to the control case (range in the case of no parameter change - case A in Figure S3.1A,B), this measured range decreases sharply in several cases – decreased production rate of let-7 (case C2, Figure S3.1A), increased degradation rate of let-7 (case D1, Figure S3.1A), decreased threshold level of miR-200 for inhibiting LIN28 (case F2, Figure S3.1A), increased threshold level of let-7 for self-activation (case G1, Figure S3.1B), increased threshold level of LIN28 for self-activation (case I1, Figure S3.1B), and decreased threshold level of LIN28 for inhibiting let-7 (case M2, Figure S3.1B). All these cases, except the case G1, lead to lower levels of let-7 in the dynamical system; therefore, the factors that can directly lower let-7 levels in the system reduce the likelihood of the circuit to exhibit tristability, or that appropriate let-7 levels are crucial for the D/U state to exist robustly.

Next, we perform the same sensitivity analysis when the LIN28/let-7 circuit is driven by miR-200 and the fixed level of NF-κB = 35000 molecules. Figure 4.4C shows that at NF-κB = 35000 molecules, range of miR-200 for the existence of D/U state almost entirely overlaps with miR-200 levels that are associated with the hybrid E/M phenotype; thereby implying that NF-κB promotes the correspondence of the D/U state with the hybrid E/M phenotype (5000-15000 molecules). Figure S3.2A, B show that for most parameter changes (either increase or decrease), D/U still overlaps largely with E/M, thereby suggesting that our prediction regarding the role of NF-κB is quite robust. Also, NF-κB rescues the stand-alone LIN28/let-7 circuit of its sensitivity towards lower let-7 levels (cases C2, D1 in Figure S3.2A).
Figure S3.1: Sensitivity analysis of LIN28/let-7 circuit driven by miR-200. Both A) and B) show the levels of miR-200 for which the D/U state exists when a particular parameter is increased or decreased by 20%. Alphanumeric codes on the x-axes represent the cases of varying different parameters. A represents the case for parameters in Fig 2B (control case). B1 and B2 represent the case for 20% increase and decrease in production rate (depicted by $g_m$) or translation rate (depicted by $g_B$) of LIN28 mRNA respectively. C1 and C2 represent the case for 20% increase and decrease in let-7 production rate (depicted by $g_\mu$) respectively. D1 and D2 represent the case for 20% increase and decrease in let-7 degradation rate (depicted by $k_\mu$) respectively. E1 and E2 represent the case for 20% increase and decrease in LIN28 degradation rate (depicted by $k_B$) or LIN28 mRNA degradation rate (depicted by $k_m$) respectively. F1 and F2 represent the case for 20% increase and decrease in the threshold level of miR-200 for LIN28 inhibition respectively. G1 and G2 represent the case for 20% increase and decrease in threshold level of let-7 for its self-activation (depicted by $\mu^{0}_\mu$) respectively. H1 and H2 represent the case for 20% increase and decrease in weight factor of shifted Hill function for self-activation of let-7 (depicted by $\lambda_{\mu,\mu}$) respectively. I1 and I2 represent the case for 20% increase and decrease in threshold level of LIN28 for its self-activation (depicted by $B^{0}_B$) respectively. J1 and J2 represent the case for 20% increase and decrease in weight factor of shifted Hill function for self-activation of LIN28 (depicted by $\lambda_{B,B}$) respectively. K1 and K2 represent the case for 20% increase and decrease in threshold level of LIN28 inhibition (depicted by $\mu^{0}_B$). L1 and L2 represent the case for 20% increase and decrease in weight factor of shifted Hill function for the inhibition of LIN28 by let-7 by LIN28 (depicted by $\lambda_{\mu,B}$) respectively. M1 and M2 represent the case for 20% increase and decrease in threshold levels of LIN28 for inhibition of let-7 (depicted by $B^{0}_\mu$) respectively. N1 and N2 represent the case for 20% increase and decrease in weight factor of shifted Hill function for the inhibition of let-7 by LIN28 (depicted by $\lambda_{B,\mu}$) respectively. Dotted red rectangles highlight cases for which the miR-200 range of existence of D/U state is the least.
Figure S3.2: Sensitivity analysis of LIN28/let-7 circuit driven by miR-200, at fixed levels of NF-κB=35000 molecules. Both A) and B) show the levels of miR-200 for which the D/U state exists when a particular parameter is increased or decreased by 20%. Alphanumeric codes on the x-axes represent the cases of varying different parameters. A represents the case for parameters in Fig 4B (control case) for NF-κB=35000 molecules. B1 and B2 represent the case for 20% increase and decrease in production rate (depicted by $g_m$) or translation rate (depicted by $g_B$) of LIN28 mRNA respectively. C1 and C2 represent the case for 20% increase and decrease in let-7 production rate (depicted by $g_\mu$) respectively. D1 and D2 represent the case for 20% increase and decrease in let-7 degradation rate (depicted by $k_\mu$) respectively. E1 and E2 represent the case for 20% increase and decrease in LIN28 degradation rate (depicted by $k_B$) or LIN28 mRNA degradation rate (depicted by $k_m$) respectively. F1 and F2 represent the case for 20% increase and decrease in the threshold level of miR-200 for LIN28 inhibition respectively. G1 and G2 represent the case for 20% increase and decrease in threshold level of NF-κB for let-7 activation (depicted by $N^0_\nu$) respectively. H1 and H2 represent the case for 20% increase and decrease in threshold level of NF-κB for LIN28 activation (depicted by $N^0_B$) respectively. I1 and I2 represent the case for 20% increase and decrease in threshold level of let-7 for its self-activation (depicted by $\mu_0^0$) respectively. J1 and J2 represent the case for 20% increase and decrease in weight factor of shifted Hill function for self-activation of let-7 (depicted by $\lambda_{\mu,\mu}$) respectively. K1 and K2 represent the case for 20% increase and decrease in threshold level of LIN28 for its self-activation (depicted by $B^0_B$) respectively. L1 and L2 represent the case for 20% increase and decrease in weight factor of shifted Hill function for self-activation of LIN28 (depicted by $\lambda_{B,B}$) respectively. M1 and M2 represent the case for 20% increase and decrease in threshold level of let-7 for LIN28 inhibition (depicted by $\mu_B^0$). N1 and N2 represent the case for 20% increase and decrease in weight factor of shifted Hill function for the inhibition of LIN28 by let-7 by LIN28 (depicted by $\lambda_{\mu,B}$) respectively. O1 and O2 represent the case
for 20% increase and decrease in threshold levels of LIN28 for inhibition of let-7 (depicted by $B_B$) respectively. P1 and P2 represent the case for 20% increase and decrease in weight factor of shifted Hill function for the inhibition of let-7 by LIN28 (depicted by $\lambda_{B,\mu}$) respectively. Red lines mark the range of miR-200 levels for the existence of the hybrid E/M phenotype (5000-15000 molecules).

Next, we test how sensitive to parameter selection is our prediction that D/U state is more likely to attain stemness as compared to the U state. We plot the range of miR-200 levels for which D/U state adopts stemness (Figures S3.3A, C) and those for which U state gains stemness (Figure S3.3B, D) for ± 20% change in parameters. For most cases, the D/U state gains stemness for a much larger range of miR-200 levels as compared to that for the U state (compare corresponding cases in Figures S3.3A and C, and Figures S3.3 B and D), reflecting its higher likelihood to gain stemness. However, specifically for four cases (C2, D1, I1, O2; Figure S3.3), both D/U and U states have a comparable range of miR-200 levels (not necessarily the same absolute levels of miR-200) to adopt stemness, and in other words, in these four cases, both states have almost equal likelihood to gain stemness. Interestingly, all these four cases represent factors that can directly lower let-7 levels in the system- C2 (decreased production rate of let-7), D1 (increased degradation rate of let-7), I1 (increased threshold level of let-7 for self-activation) and O2 (decreased threshold level of LIN28 for the Hill function representing let-7 inhibition by LIN28). Therefore, this implies that our prediction about D/U state being likely to gain stemness is robust to all parameters, however, the prediction that stemness is associated with D/U state more strongly than U state is sensitive towards parameters that tend to lower let-7 levels in the system, or, very low let-7 levels make the D/U and U states both almost equally likely to gain stemness.
Figure S3.3: Sensitivity analysis of stemness of D/U and U states for LIN28/let-7 circuit driven by miR-200, at fixed levels of NF-κB=25000 molecules. Both A) and C) show the levels of miR-200 for which the D/U state had stemness when a particular parameter is increased or decreased by 20%. Both B) and D) show the levels of miR-200 for which the D/U state had stemness when a particular parameter is increased or decreased by 20%. Alphanumeric codes on the x-axes represent the cases of varying different parameters. A represents the case for parameters in Fig 4B (control case) for NF-κB=35000 molecules. B1 and B2 represent the case for 20% increase and decrease in production rate (depicted by $g_m$) or translation rate (depicted by $g_B$) of LIN28 mRNA respectively. C1 and C2 represent the case for 20% increase and decrease in let-7 production rate (depicted by $g_\mu$) respectively. D1 and D2 represent the case for 20% increase and decrease in let-7 degradation rate (depicted by $k_\mu$) respectively. E1 and E2 represent the case for 20% increase and decrease in LIN28 degradation rate (depicted by $k_B$) or LIN28 mRNA degradation rate (depicted by $k_m$) respectively. F1 and F2 represent the case for 20% increase and decrease in the threshold level of miR-200 for LIN28 inhibition respectively. G1 and G2 represent the case for 20% increase and decrease in threshold level of NF-κB for let-7 activation (depicted by $N_\mu^0$) respectively. H1 and H2 represent the case for 20% increase and decrease in threshold level of NF-κB for LIN28 activation (depicted by $N_B^0$) respectively. I1 and I2 represent the case for 20% increase...
and decrease in threshold level of let-7 for its self-activation (depicted by $\mu_0^\mu$) respectively. J1 and J2 represent the case for 20% increase and decrease in weight factor of shifted Hill function for self-activation of let-7 (depicted by $\lambda_{\mu,\mu}$) respectively. K1 and K2 represent the case for 20% increase and decrease in threshold level of LIN28 for its self-activation (depicted by $B_0^\mu$) respectively. L1 and L2 represent the case for 20% increase and decrease in weight factor of shifted Hill function for self-activation of LIN28 (depicted by $\lambda_{B, B}$) respectively. M1 and M2 represent the case for 20% increase and decrease in threshold level of let-7 for LIN28 inhibition (depicted by $\mu_0^B$). N1 and N2 represent the case for 20% increase and decrease in weight factor of shifted Hill function for the inhibition of LIN28 by let-7 by LIN28 (depicted by $\lambda_{\mu, B}$) respectively. O1 and O2 represent the case for 20% increase and decrease in threshold levels of LIN28 for inhibition of let-7 (depicted by $B_0^\mu$) respectively. P1 and P2 represent the case for 20% increase and decrease in weight factor of shifted Hill function for the inhibition of let-7 by LIN28 (depicted by $\lambda_{B, \mu}$) respectively. Dotted red rectangles mark the cases (C2, D1, I1, O2) when D/U and U states have comparable likelihood to adopt stemness. For cases where no range is marked for stemness (cases C1, D2, I2, O1), the U state was not found to gain stemness.

Finally, we perform the sensitivity analysis when LIN28/let-7 circuit is driven by miR-200 and c-MYC. We calculate the minimum values of c-MYC for which the LIN28/let-7 becomes monostable (i.e. only U state exists) and find that for most parameter changes (either increase or decrease), these values are less than the typical values of c-MYC in a cell (~28000 molecules) (Figure S3.4A, B), thereby suggesting that our prediction that c-MYC drives the cells to a U or (1, 0) state is largely robust to parameters. Also, c-MYC rescues the sensitivity of the stand-alone circuit towards lower let-7 levels.

In conclusion, NF-kB and c-MYC robustly rescues the sensitivity of the stand-alone LIN28/let-7 circuit to lower let-7 levels. Also, predictions about c-MYC and NF-kB driving the system to U and D/U state correspondingly are robust to variations. Lastly, for most cases of parameter variation, NF-kB promotes the association of stemness with D/U state much more that with the U state; therefore, D/U state’s association with stemness is robustly promoted by NF-kB.
Figure S3.4: Sensitivity analysis of LIN28/let-7 circuit driven by miR-200 and c-MYC. Both A) and B) show the levels of miR-200 for which the D/U state exists when a particular parameter is increased or decreased by 20%. Alphanumeric codes on the x-axes represent the cases of varying different parameters. A represents the case for parameters in Fig 3A. B1 and B2 represent the case for 20% increase and decrease in production rate (depicted by $g_m$) or translation rate (depicted by $g_B$) of LIN28 mRNA respectively. C1 and C2 represent the case for 20% increase and decrease in let-7 production rate (depicted by $g$) respectively. D1 and D2 represent the case for 20% increase and decrease in let-7 degradation rate (depicted by $k_m$) respectively. E1 and E2 represent the case for 20% increase and decrease in threshold level of miR-200 for LIN28 inhibition (depicted by $C_{m0}$) respectively. H1 and H2 represent the case for 20% increase and decrease in threshold level of c-MYC for LIN28 activation (depicted by $C_B^0$) respectively. I1 and I2 represent the case for 20% increase and decrease in threshold level of let-7 for its self-activation (depicted by $\mu^0$) respectively. J1 and J2 represent the case for 20% increase and decrease in threshold level of let-7 for LIN28 inhibition (depicted by $\mu^0_B$). N1 and N2 represent the case for 20% increase and decrease in weight factor of shifted Hill function for the inhibition of LIN28 by let-7 by LIN28 (depicted by $\lambda_{\mu,B}$) respectively. O1 and O2 represent the case for 20% increase and decrease in threshold levels of LIN28 for inhibition of let-7.
(depicted by $B_0$) respectively. P1 and P2 represent the case for 20% increase and decrease in weight factor of shifted Hill function for the inhibition of let-7 by LIN28 (depicted by $\lambda_{B,\mu}$) respectively.

Effect of c-MYC

When driven by both miR-200 and c-MYC, and miR-200 levels are greater than those that correspond to the hybrid E/M phenotype (i.e. >15000 molecules (97), the range of c-MYC for which the D/U state exists decreases significantly (Figure S3.5A) as compared to the same range of c-MYC when miR-200 levels are in between 5000-15000 molecules (Figure 4.3A). For even higher values of miR-200, the circuit becomes bistable. Also, when miR-200 levels are smaller than those that correspond to the hybrid E/M phenotype (i.e. <5000 molecules (97)), the circuit can be monostable, and only the U state exists (Figure S3.5B).

Figure S3.5: Circuit response to c-MYC and miR-200. Bifurcation diagram showing the dependence of the level of LIN28 on c-MYC when LIN28 is inhibited by (A) constant miR-200 level=20000 molecules, and (B) constant miR-200 level=2000 molecules. Blue solid lines indicate stable states, and red dotted lines denote unstable states. Corresponding EMT phenotypes have been shown for every stable state.

Materials and Methods

Model simulation

The bifurcation plots were evaluated using MATCONT(284).
Appendix 4: Clusters of cells in a hybrid E/M phenotype

Model formulation for coupled EMT-Notch regulatory circuit

The equations governing the levels of Notch(N), Delta (D), Jagged (J) and cleaved Notch, i.e. NICD (I) are given by:

\[
\frac{dN}{dt} = k_p g_N H^S(I, N) P_l(\mu_{34}, 2) - N[(k_{cD}D + k_{cJ}J) + (k_{tD}D_{ext} + k_{tJ}J_{ext})] - \gamma N \tag{S1}
\]

\[
\frac{dD}{dt} = k_p g_D H^S(I, D) P_l(\mu_{34}, 3) - D(k_{cD}N + k_{tD}N_{ext}) - \gamma D \tag{S2}
\]

\[
\frac{dJ}{dt} = k_p g_J H^S(I, J) P_l(\mu_{200}, 5) - J(k_{cJ}N + k_{tJ}N_{ext}) - \gamma J \tag{S3}
\]

\[
\frac{dI}{dt} = N(k_{tD}D_{ext} + k_{tJ}J_{ext}) - \gamma I \tag{S4}
\]

where \(k_{cD}\) and \(k_{cJ}\) are cis-inhibition (mutual inactivation resulting from binding between Notch and Delta/Jagged of a cell (232, 244)) rates for Notch-Delta and Notch-Jagged signaling respectively, and \(k_{tD}\) and \(k_{tJ}\) are respective trans-activation (binding between Notch and Delta/Jagged of neighboring cells, leading to NICD cleavage (232)) rates. These rates are a function of NICD (I) because both cis-inhibition and trans-activation rates are modulated by glycosyltransferase Fringe that is induced by NICD (242, 243, 291, 292), and are given by: \(k_{cD} = k_c H^S(I, \lambda_{F,D})\), \(k_{tD} = k_c H^S(I, \lambda_{F,D})\), \(k_{cJ} = k_c H^S(I, \lambda_{F,J})\), \(k_{tJ} = k_c H^S(I, \lambda_{F,J})\). Since Fringe increases the binding affinity between Notch and Delta and decreases that between Notch and Jagged, \(\lambda_{F,J} < 1\) and \(\lambda_{F,D} > 1\). \(\gamma\) represents the degradation rate of Notch, Delta, and Jagged, \(\gamma_I\) denotes the degradation rate for NICD, \(k_p\) denotes the translation rate, and \(g_N, g_D, g_J\) represent the transcription rate. \(N_{ext}, D_{ext}, J_{ext}\) represent the amount of protein available for binding – either on the membrane of neighboring cell or in a soluble form. The modulation of production rate of Y by X is considered via shifted Hill functions denoted by \(H^S(X,Y)\); \(P_l(\mu,n)\) denotes the translational inhibition function (as defined in Fig S1.1A) due to the binding of the target mRNA with n binding sites of miRNA (97).
\[ \frac{d\mu_{200}}{dt} = g_{\mu_{200}} H^S(Z, \mu_{200}) H^S(S, \mu_{200}) - g_Z H^S(Z, Z) H^S(S, Z) P_y(\mu_{200}, 6) \]
\[ - g_J H^S(I, J) P_y(\mu_{200}, 5) - \gamma_{\mu_{200}} \mu_{200} \]  
(S5)

\[ \frac{d\mu_{34}}{dt} = g_{\mu_{34}} H^S(S, \mu_{34}) H^S(Z, \mu_{34}) - g_S H^S(S, S) H^S(I, S) H^S(I_{ext}, S) P_y(\mu_{34}, 2) \]
\[ - g_D H^S(I, D) P_y(\mu_{34}, 3) - g_N H^S(I, N) P_y(\mu_{34}, 2) - \gamma_{\mu_{34}} \mu_{34} \]  
(S6)

\[ \frac{dZ}{dt} = k_P g_Z H^S(Z, Z) H^S(S, Z) P_I(\mu_{200}, 6) - \gamma_Z Z \]  
(S7)

\[ \frac{dS}{dt} = k_P g_S H^S(S, S) H^S(I, S) H^S(I_{ext}, S) P_I(\mu_{34}, 2) - \gamma_S S \]  
(S8)

(S5)-(S8) are the equations for EMT circuit, i.e. miR-200 (\(\mu_{200}\)), miR-34 (\(\mu_{34}\)), SNAIL (\(S\)), ZEB (\(Z\)). \(g_{\mu_{200}}, g_{\mu_{34}}, g_Z, g_S\) are respective production rates for miR-200, miR-34, ZEB and SNAIL; while \(\gamma_{\mu_{200}}, \gamma_{\mu_{34}}, \gamma_Z, \gamma_S\) are their respective innate degradation rates. \(I_{ext}\) represents an external signal that induces EMT by activating SNAIL. For the case of many interacting cells, the variables \(N_{ext}, D_{ext}, J_{ext}\) are replaced by \(N, D, J\) of neighboring cells, as discussed in detail in our previous work (235, 236).

**Parameter values for coupled EMT-Notch regulatory circuit**

For a detailed discussion of parameters for EMT circuit, please see Appendix 1.2 or (97), that about the parameters for Notch circuit is given in our previous work (235, 236). For most parameters, the values used are those mentioned in original references. Only a few parameter values were adjusted as mentioned below in Table S4.1. Values of \(g_N, g_D, g_J\) were chosen in order to keep the maximum number of proteins in membrane up to a few thousand molecules/cell, which is consistent with the concentration of proteins varying up to a few hundred ng/ml(293). Similarly, the number of NICD molecules inside the nucleus can vary up to a few hundred molecules, hence the threshold of NICD (\(I_0\)) has been chosen to be 200 for modulation of Notch, Delta and Jagged, and 300 for the activation of SNAIL. A smaller Hill coefficient for Jagged was chosen as compared to our previous work because now the strong indirect activation of Jagged by
NICD via miR-200 is already considered in this framework (235). Translation rate of all proteins is chosen to be 100 proteins per mRNA per hour. Also, Notch signaling dynamics is assumed to be considerably slower than EMT dynamics, hence the values of cis-inhibition and trans-activation have been considered to be 5 times slower than what used previously \((k_T = 1e^{-5}, k_C = 1e^{-4})\) instead of \((k_T = 5e^{-5}, k_C = 5e^{-4})\)(235). Sensitivity analysis for EMT circuit is discussed in detail in Appendix 2, that for the Notch circuit is shown in Figure S4.10. \(n_N, n_D, n_J, n_S, \lambda_{I,N}, \lambda_{I,D}, \lambda_{I,J}, \lambda_{I,S}\) denote the respective Hill coefficient and fold-change for shifted Hill functions showing the effect of NICD on Notch, Jagged, Delta and SNAIL.

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**Table S4.1: Parameter values for coupled Notch-EMT circuit.** The symbol * denotes the values used for 1 cell simulations (triggered by external Notch, Delta, or Jagged) for Figure 2, and ** denotes the values used in Figure 3.

**Materials and Methods**

**Cell culture** MCF10A cells were maintained in DMEM/F12 media (SigmaAldrich) supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 mg/ml insulin and penicillin/streptomycin (1%). To induce EMT, they were treated with vehicle or 5 ng/ml of TGF-b1 (R&D systems) for 6 days. MDA-MB-231 and MDA-MB-468 cells (ATCC) were cultured in DMEM containing 10% fetal bovine serum at 37°C and 5% CO2. During treatments with chemotherapeutics, cells were grown to semi-confluency and treated with indicated concentrations of chemotherapy in serum-containing medium for indicated time points. For generation of DTCs, cells were treated for 48 h with docetaxel (100 nM). Following washes with phosphate-buffered saline (PBS), adherent cells were trypsinized and re-plated at a density of 1.5 – 2 105 cells/ml and cultured in serum-containing medium onto glass slides (BD, San Jose, CA). After 24 h incubation, floating cells were removed and remaining cells were washed with 1 PBS and considered as chemotherapy-tolerant cells. Populations of drug naive parent cells were always cultured alongside DTC and fresh media was added at every interval that the experimental population (DTC) received fresh media. Unless noted otherwise, all reagents and chemotherapies were of the highest grade.
purchased from Sigma-Aldrich (St Louis, MO, USA). All chemotherapeutics were dissolved in dimethylsulfoxide to a stock concentration of 10 mM and kept frozen before fresh preparation into working concentration in DMEM. 4.3.

**Confocal microscopy and immunofluorescence** Parent cells or DTCs were generated as described above and plated in four chamber glass slides (BD Biosciences, San Jose, CA, USA) at a concentration of 10 000 cells ml⁻¹. Following treatments, cells were washed in PBS and fixed in 4% paraformaldehyde for 30 min. Permeabilization, when necessary, was achieved with 10% (v/v) goat serum (Vector Laboratories, Burlingame, CA, USA) and 0.05% Saponin (w/v) in PBS for 90 min. Blocking was performed in 10% (v/v) goat serum in PBS. The cells were labelled with the indicated fluorescently conjugated primary antibodies CD44 (Clone IM7 from eBioScience) at 1 : 500, CD24 (clone ML5 from eBioScience) at 1 : 100, Jagged-1 (cat# 200-401-698S from Rockland, Limerick, PA, USA), Delta at 1 : 100 (clone H-265 from Santa Cruz Biotech, Dallas, TX, USA), cleaved notch at 1 : 50 (clone ab8925 from Abcam, Cambridge, MA, USA) for 24 h at 48°C and masked with hard-set mounting medium (Vector Laboratories, Burlingame CA). Bright field and fluorescent images were obtained using three channels on a Nikon Eclipse TI-U microscope with a 20 ELDW, 10 or 40 Plan-Apo objective lens (Nikon, Melville, NY, USA). NIS Elements Viewer version 3.22 (Nikon) software was used to capture the images to file. Confocal microscopy of IHC from frozen sections of tumour tissue was performed with an inverted Nikon confocal microscope (TE2000) with Auto DeBlur deconvolution software and fitted with three laser detection (Nikon). Gains were set manually based on negative control stains (secondary antibody only) and were left unaltered between treatment groups of similar experiments. When representative images are shown in figures, these are derived from experiments performed in at least biological triplicate on independent occasions. Quantification of the fluorescent intensity was determined using Adobe CS5 software (San Jose, CA, USA) and confirmed using ImageJ software (NIH) and indication of CD44 Hi/Lo or CD24Hi/Lo was determined by relative fluorescent intensities between individual cells. For immunofluorescence staining of MCF10A cells, they were plated on pre-sterilized coverslips and were fixed and permeabilized with 4% parafomaldehyde þ 0.1% Triton-X 100 for 10 min at room temperature (RT). PFA was quenched by 5% glycine 15 min RT and samples were blocked with 4% bovine serum albumin (BSA) in PBS for 1 h RT. Primary antibody against cleaved Notch-1 (NICD, Cell Signaling Technology) was diluted 1:1000 in 4% BSA in PBS and incubated overnight at 48°C. Species-specific Alexa Fluor 488—conjugated secondary antibody (Life Technologies) was diluted 1 : 1000 in 4% BSA in PBS and incubated 1 h RT. Nuclei were counterstained with 40 ,6-diamidino-2-phenylindole (DAPI; Molecular Probes). The coverslips were mounted onto glass slides with DAKO fluorescent mounting medium (DAKO). 4.4.

**Statistics** Statistical analysis was performed using Prism software (Graphpad, La Jolla, CA determined by ANOVA followed by a Newman–Keuls post hoc test when values were represented between multiple groups and Student’s t-test used to identify statistical significance between individual groups. The data are expressed as a mean+s.e.m.
**Theoretical framework** Bifurcation diagrams were evaluated using PyDSTool(294).

**Supplementary figures**

Figure S4.1: Bifurcation curves of the levels of SNAIL, ZEB, miR-34, and miR-200 in response to external signal ($I_{ext}$) that activates SNAIL
Figure S4.2: Bifurcation curves of the levels of SNAIL, ZEB, miR-34, and miR-200 in response to external Delta ($D_{ext}$) (a) and external Jagged ($J_{ext}$) (b).

Figure S4.3: Initial condition for the simulations presented in Figures 4, S4.4 and S4.7. The levels of each protein is chosen randomly from a uniform distribution where the range is defined based on the maximum and minimum values presented in Figure S3. A) Color represents the levels of miR200. B) Color represents the cell state: (E: miR200 > 15000, E/M: 15000 > miR200 > 5000, M: miR200 < 5000 molecules)
Figure S4.4: Temporal evolution starting from the initial condition shown in Figure S4.3. Left column represents higher production of Jagged, right column represents higher production of Delta. A) No external signal lead to transient clusters, B) Cells can maintain a salt-and-pepper patterning between epithelial and hybrid E/M cells even in absence of any external signal. C) EMT-inducing signal stabilizes the clusters potentially and drive the hybrid E/M cells to a complete EMT. D) EMT-inducing signal stabilizes salt-and-pepper pattern for epithelial and hybrid E/M cells. E) External soluble Jagged can stabilize the clusters and increase the number of cells in hybrid E/M phenotype. F) External soluble Jagged disrupts the salt-and-pepper patterning and can initiate reorganization of hybrid E/M cells to form clusters.

Figure S4.5: Effect of external inducers of the Notch-EMT coupled circuit on tissue patterning. Simulation of a 2D layer of 50x50 cells interacting via Notch-Delta-Jagged signaling. A) Fraction of cells for epithelial (E), epithelial/mesenchymal (E/M) and mesenchymal (M) phenotypes for different levels of an EMT-inducer signal ($I_{\text{ext}}$). B) Snapshot of simulated tissue representing the spatial distribution of E, E/M and M cells for $I_{\text{ext}} = 70$ molecules, $g_D = 70$ and $g_J = 20$ molecules/h. C) Fraction of cells for each phenotype for different levels of external soluble Jagged ($sJ_{\text{ext}}$) for $I_{\text{ext}} = 0$ molecules, $g_D = 20$ and $g_J = 70$ molecules/h. D) Snapshot of simulated tissue representing the spatial distribution of E, E/M and M cells $sJ_{\text{ext}} = 4000$ molecules. The levels were measured after an equilibrium time of 120h, starting from the configuration presented in Figure 5.4C.
Figure S4.6: Effect of soluble Delta on tissue patterning. Simulation of a 2D layer of 50x50 cells interacting via Notch-Delta-Jagged signaling. A) Fraction of cells for each phenotype: epithelial (E), epithelial/mesenchymal (E/M) and mesenchymal (M) for different levels of soluble Delta (sDext), for gD = 20 and gJ = 70 molec/h. B) Snapshot of the simulated tissue representing the spatial distribution of E, E/M and M cells for Dext = 1000 molecules, gD = 20 and gJ = 70 molecules/h. C) same as A) for gD = 70 and gJ = 20 molecules/h. D) Snapshot of the simulated tissue representing the spatial distribution of E, E/M and M cells for sDext = 1000 molecules, gD = 70 and gJ = 20 molecules/h.
**Figure S4.7: Effect of Fringe on tissue patterning.** Simulation of a 2D layer of 50x50 cells interacting via Notch-Delta-Jagged signaling, starting from the initial condition described in Figure S4 after 120h. A) Fraction of cells for each phenotype: epithelial (E), epithelial/mesenchymal (E/M) and mesenchymal (M) for different values of Fringe effect (fng), for gD = 20 and gJ = 70 molec/h. B) Snapshot of the simulated tissue representing the spatial distribution of E, E/M and M cells for the case of no Fringe effect (fng = 0.0, i.e, λ F D = λ F J = 1.0), gD = 20 and gJ = 70 molec/h. Compare with figure 4D (intermediate Fringe effect). C) Same as A) for gD = 70 and gJ = 20 molec/h. D) Snapshot of the simulated tissue representing the spatial distribution of E, E/M and M cells for the case of no Fringe effect (fng = 0.0, i.e, λ F D = λ F J = 1.0), gD = 70 and gJ = 20 molec/h. Compare with figure 4C (intermediate Fringe effect). As fng increases, the values of λ F D and λ F J linearly increase and decrease respectively, such that at fng = 1.0, λ F D = 5.0 and λ F J = 0.1, i.e. Notch has higher binding affinity to Delta and lower to Jagged.

**Figure S4.8: Simulation of 50x50 cells interacting via N-D-J signaling for g_D = 70 and g_J = 20 molecules/h.** (middle) Fraction of cells adopting Epithelial (E), Epithelial/Mesenchymal (E/M) and Mesenchymal (M) phenotypes at different time
points for the given initial condition. (left, right) Levels of miR-200 for 50x50 hexagonal lattice at t=0 and t=360 hr. Red cells are in an M phenotype, yellow ones in a hybrid E/M phenotype and green ones in the E phenotype. A) Starting from 90% of cells in the M state and 10% in the E/M state. B) Starting from 70% of cells in the M state and 30% in the E/M state. C) Starting from 50% of cells in the M state and 50% in the E/M state.

Figure S4.9: Same as Figure S4.8 but for $g_D = 20$ and $g_J = 70$ molecules/h.

**Sensitivity analysis for Notch-Delta-Jagged circuit**

In our work focusing on developing a theoretical framework to capture Notch-Delta-Jagged dynamics, we varied every parameter in the model by 10% (both increase and decrease) and investigated the changes in NICD steady state levels for (a) only Notch-Delta signaling, (b) Notch-Delta-Jagged signaling, and (c) Notch-Delta-Jagged-Fringe signaling. In all these cases, the most sensitive parameters are: production rate of Notch $N_0$, production rate of Delta $D_0$, trans-activation rate $k_T$, and degradation rate of Notch $\gamma_N$ (Figure S4.10). To evaluate the influence of these four parameters in the shape of the bifurcations curves, we changed by 10% the values of each parameter. The limit point where the system changes from one state to the other was found to be very sensitive
to changes in these parameters (235); however, the overall behavior of the circuit remains the same, therefore suggesting a good robustness of the model.

Figure S4.10: Sensitivity analysis of Notch-Delta, Notch-Delta-Jagged, and Notch-Delta-Jagged-Fringe circuits. The graph shows relative changes in levels of NICD steady state levels in response to variation of parameter values by 10% (both increase and decrease). The parameters used here are mentioned in Table S1 of our previous work (235).
References


42. Schliekelman MJ, et al. (2015) Molecular portraits of epithelial, mesenchymal and


82. Liotta LA, Klelnerman J, Saldel GM (1976) The significance of hematogenous


126. Zhou JX, Huang S (2011) Understanding gene circuits at cell-fate branch points...


Subpopulation that Redefines Global Cancer Features. *Front Oncol* 3(April):76.


