

Population heterogeneity in the epithelial to mesenchymal transition is controlled by NFAT and phosphorylated Sp1

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Abstract

Epithelial to mesenchymal transition (EMT) is an essential differentiation program during tissue morphogenesis and remodeling. EMT is induced by soluble transforming growth factor β (TGF- β) family members, and restricted by vascular endothelial growth factor family members. While many downstream molecular regulators of EMT have been identified, these have been largely evaluated individually without considering potential crosstalk. In this study, we created an ensemble of dynamic mathematical models describing TGF- β induced EMT to better understand the operational hierarchy of this complex molecular program. These models incorporate mass action kinetics within an ordinary differential equation (ODE) framework to describe the transcriptional and post-translational regulatory events driving EMT. Model parameters were estimated from multiple data sets using multiobjective optimization, in combination with cross-validation. TGF- β exposure drove the model population toward a mesenchymal phenotype, while an epithelial phenotype was maintained following vascular endothelial growth factor A (VEGF-A) exposure. Simulations predicted that the transcription factors phosphorylated SP1 and NFAT were master regulators promoting or inhibiting EMT, respectively. Surprisingly, simulations also predicted that a cellular population could exhibit phenotypic heterogeneity (characterized by a significant fraction of the population with both high epithelial and mesenchymal marker expression) if treated simultaneously with TGF- β and VEGF-A. We tested this prediction experimentally in both MCF10A and DLD1 cells and found that upwards of 45% of the cellular population acquired this hybrid state in the presence of both TGF- β and VEGF-A. We experimentally validated the predicted NFAT/Sp1 signaling axis for each phenotype response. Lastly, we found that cells in the hybrid state had significantly different functional behavior when compared to VEGF-A or TGF- β treatment alone. Together, these results establish a predictive mechanistic model of EMT susceptibility, and potentially reveal a novel signaling axis which regulates carcinoma progression through an EMT versus tubulogenesis response.

Author Summary

Tissue formation and remodeling requires a complex and dynamic balance of interactions between epithelial cells, which reside on the surface, and mesenchymal cells that reside in the tissue interior. During embryonic development, wound healing, and cancer, epithelial cells transform into a mesenchymal cell to form new types of tissues. It is important to understand this process so that it can be controlled to generate beneficial effects and limit pathological differentiation. Much research over the past 20 years has identified many different molecular species that are relevant, but these have mainly been studied one at a time. In this study, we developed and implemented a novel computational strategy to interrogate all of the known players in this transformation process to identify which are the major bottlenecks. We determined that NFATc1 and pSP1 are essential for promoting epithelial or mesenchymal differentiation, respectively. We then predicted the existence of a partially transformed cell that exhibits both epithelial and mesenchymal characteristics. We found this partial cell type develops a network of invasive but stunted vascular structures that may be a unique cell target for understanding cancer progression and angiogenesis.

Introduction

The epithelial to mesenchymal transition (EMT) is a broadly participating, evolutionarily conserved differentiation program essential for tissue morphogenesis, remodeling and pathological processes such as cancer [1]. During EMT polarized, tightly adhered epithelial cell monolayers are transformed into non-interacting motile mesenchymal cells that simultaneously degrade and synthesize extracellular matrix (ECM) components and invade into the underlying tissue space [2]. EMT is the fundamental initiator of developmental processes such as embryonic gastrulation and valvulogenesis [3] (also Kalluri J Clin Invest 2009, Thiery Cell 2009). Transforming growth factor β (TGF- β) family members are important inducers of both developmental and pathological EMT [4,5]. Decades of research has focused on identifying molecular regulators of EMT, but almost all on a single gene and in a nearly binary yes/no level of qualitative understanding. Medici and coworkers recently identified a core signaling program by which TGF- β isoforms induce EMT across a variety of cell lines [6,7]. This program involves carefully orchestrated rounds of gene expression driven by the Smad and Snail families of transcription factors as well as other key factors such as lymphoid enhancer-binding factor 1 (LEF-1), nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), and specificity protein 1 (Sp1). Coregulators such as β -catenin, NF- κ B, and the ErbB family of receptor tyrosine kinases however also participate in EMT regulation, but the degree of each's influence is difficult to ascertain in isolation [8–11]. EMT also exhibits complex temporal dynamics that are often intractable in gain/loss of function studies. Elucidating the master regulatory architecture controlling EMT therefore requires inclusion of these complex overlapping and non-binary

behaviors.

Systems biology and mathematical modeling are essential tools for understanding complex developmental programs like EMT [12]. Previous computational models of TGF- β induced differentiation focused on single biological factors or EMT in single cells. For example, Chung *et al.*, constructed a model of TGF- β receptor activation and Smad signaling using ordinary differential equations and mass-action kinetics. Their model suggested that a reduction of functional TGF- β receptors in cancer cells may lead to an attenuated Smad2 signal [13]. Similarly, Vilar *et al.* suggested that specific changes in receptor trafficking patterns could lead to phenotypes that favor tumorigenesis [14]. Although these models provided insight into the role of receptor dynamics, EMT induction involves many other components, including competing second messengers and interconnected transcriptional regulatory loops. Integrating these additional scales of molecular signaling while maintaining the capacity for robust prediction requires a new and expanded computational and experimental strategy. Data-driven systems approaches [15] or logical model formulations [16] are emerging paradigms that constrain model complexity through the incorporation of training and validation data. These are interesting techniques because the data informs model structure (which can be expanded as more data becomes available). Alternatively, Bailey proposed more than a decade ago that a qualitative understanding of a complex biological system should not require complete definition of its structural and parametric content [17]. Shortly thereafter, Sethna and coworkers showed that complex model behavior is often controlled by only a few parameter combinations, a characteristic seemingly universal to multi-parameter models referred to as “sloppiness” [18]. Thus, reasonable model predictions are often possible with only limited parameter information. Taking advantage of this property, we developed sloppy techniques for parameter identification using ensembles of deterministic models [19]. Furthermore, we proposed that the sloppy behavior of biological networks may also be seen as a source of cell-to-cell [20] or even patient-to-patient heterogeneity [21]. Recently, Bayesian parameter identification techniques have also been used to explore cell-to-cell heterogeneity [22, 23], where a population of cells could be viewed as a dynamic ensemble of context-specific biochemical networks [24].

In this study, we developed a family of mechanistic models describing the induction of EMT by TGF- β isoforms in the presence and absence of vascular endothelial growth factor A (VEGF-A). We incorporated mass action kinetics within an ordinary differential equation (ODE) framework to describe the EMT interaction network containing 995 gene, protein or mRNA components interconnected through 1700 interactions. A family of model parameters was estimated using 41 molecular data sets generated in DLD1 colon carcinoma, MDCKII and A375 melanoma cells using the Pareto optimal ensemble technique (POETs) multiobjective optimization algorithm. POETs identified more than 15,000 likely TGF- β induced EMT models, from which we selected approximately 1100 models for further analysis. Analysis of the model population suggested that both MCF10A and DLD1 cells could exhibit

phenotypic heterogeneity if treated simultaneously with TGF- β 1/2 and VEGF-A. This heterogeneity was characterized by a significant fraction of the population being in a “hybrid state” having both high E-cadherin and high Vimentin expression. We tested these predictions using qRT-PCR and flow-cytometry studies in a variety of experimental conditions. Validation studies confirmed that upwards of 45% of the cellular population could be put into the hybrid state in the presence of both TGF- β 1/2 and VEGF-A. Moreover, this response depended upon both activation of Sp1 by MAPK and NFATc1 transcriptional activity consistent with the predicted molecular signaling. Lastly, the hybrid populations of both DLD1 and MCF10A cells exhibited different functional behavior than those from either TGF- β or VEGF-A treatment. The extent of ductal branch formation significantly increased with MCF10A cells in the hybrid phenotype, compared with cells treated with VEGF-A alone. Together, these results establish a predictive mechanistic model of EMT susceptibility, and reveal a novel signaling axis, which possibly regulates carcinoma progression through an EMT versus tubulogenesis response.

Results

The model population captured key features of TGF- β induced EMT

The EMT model architecture, based upon curated molecular connectivity, described the expression of 80 genes following exposure to TGF- β isoforms and VEGF-A (Fig. 1). The EMT model contained 995 molecular species interconnected by 1700 interactions. Model equations were formulated using mass-action kinetics within an ordinary differential equation (ODE) framework. ODEs and mass action kinetics are common tools to model biochemical pathways [25–27]. However, while ODE models can simulate complex intracellular behavior, they require estimates for model parameters which are often difficult to obtain. The EMT model had 1756 unknown model parameters, 1700 kinetic constants and 56 non-zero initial conditions. As expected, these parameters were not uniquely identifiable given the training data [28]. Thus, instead of identifying a single best fit (but uncertain) model, we estimated a sloppy population of models (each consistent with the training data) by simultaneously minimizing the difference between model simulations and 41 molecular data sets using the Pareto Optimal Ensemble Technique (POETs). The training data were generated in DLD1 colon carcinoma, MDCKII, and A375 melanoma cells following exposure to TGF- β isoforms [7]. We organized these data sets into 11 objective functions which were simultaneously minimized by POETs. Additionally, we used 12 molecular data sets generated in HK-2 cells following VEGF-A exposure to train VEGF-A responsive model processes [29]. To guard against overfitting, we augmented the multiobjective optimization with leave-one-out cross validation to independently estimate both the training and prediction error for each objective. Thus, we generated 11 different model ensembles. Lastly, we compared model predictions with independent data sets not used during training (both at the molecular and model population levels) to evaluate the predictive power of the parameter ensemble. Additional details of the signaling architecture included in the model are presented in the materials and methods and the supplement.

POETs generated a population of probable signaling models which captured the multiple phases of EMT induction (Fig. 2). POETs sampled well over 10^6 probable models during each stage of the cross-validation, using a combination of both local and global random sampling. This sampling generated approximately 15,000 highly probable models from which we selected $N \simeq 1100$ models for further analysis. The selected models all had the same possible molecular connectivity, but different values for model parameters and extrinsic factors such as RNA polymerase or ribosome abundance. Model selection was based upon Pareto rank, the prediction and training error across all objectives and model to model correlation (supplemental materials). The model population recapitulated key signaling events following TGF- β exposure. We subdivided the response to TGF- β exposure into two phases. First, TGF- $\beta 1/2$ signaling initiated a program which downregulated E-cadherin expression in a MAPK dependent manner while simultaneously upregulating TGF- $\beta 3$ expression.

Second, TGF- β 3 secretion initiated an autocrine feedback which upregulated the expression of mesenchymal markers such as Vimentin and key upstream transcription factors such as LEF-1 in a SMAD dependent manner. Each phase involved the hierachal expression and/or post-translational modification of several key transcription factors. During the first phase, stimulation with TGF- β 1/2 (10 a.u.) activated both the SMAD and MAPK pathways. MAPK activation resulted in the phosphorylation of the transcription factor activator protein 1 (AP-1), which in-turn upregulated the expression of Snail, a well established transcriptional repressor (Fig. 2A). Snail expression was MAPK-dependent; the MEK inhibitor U0126 blocked AP-1 activation and Snail expression following TGF- β 1/2 exposure (Fig. 2A, Lane 3). Similar results were obtained for Slug expression, confirming initial activation through the MAPK pathway (data not shown). Overexpression of either Snail or Slug upregulated TGF- β 3 expression (Fig. 2C) while simultaneously downregulating E-cadherin expression (Fig. 2F). During the second phase, TGF- β 3 secretion and the subsequent autocrine signaling resulted in the upregulation of mesenchymal marker expression. The TGF- β 3 induced gene expression program involves a complex hierarchy of transcriptional and post-translational regulatory events. Absence of E-cadherin indirectly promoted TGF- β 3 expression through the β -catenin/TCF4 complex following Snail or Slug expression (Fig. 2C, Lane 2 or 3). Conversely, over-expression of E-cadherin inhibited the TGF- β 3 autocrine production by sequestering cytosolic β -catenin, thereby blocking EMT (Fig. 2C, Lane 4 or 5). TGF- β 3 signaled through the Smad pathway to regulate LEF-1 expression and downstream target EMT genes (Fig. 2G). TGF- β 3 (10 a.u.) in combination with downstream inhibitors (DN-Smad4 and DN-LEF-1) completely inhibited Vimentin expression, while elevating E-cadherin expression (Fig. 2H,I).

The predictive power of the ensemble was tested using both cross validation and by comparing simulations with data sets not used for model training. In whole, 78% of our training objectives were statistically significant (at a 95% confidence interval) compared to a randomized parameter family ($N = 100$) generated from the best-fit nominal set (starting point for the optimization). Conversely, we *predicted* approximately 60% of the training objectives, at a 95% confidence interval compared to randomized parameters. The model also captured the temporal gene expression responses of E-cadherin, pSmad2, and LEF-1 to within one-standard deviation (up to the 48 hr time-point) (Fig. 2J-L). This data was not used for model training. The high predictability can be attributed to the combination of the leave-one-out cross validation scheme, diverse objective functions, and robustness of the POETs algorithm. Taken together, the model captured the key signaling events revealed by Medici *et al.* [7] that drive the phenotypic conversion. A listing of data used for training is included in the supplement (Fig. S5 and Fig. S6).

Identification of a novel LEF-1 regulator

During model identification, we found that consistent TGF- β induced EMT required an additional regulatory protein. This protein, which we called

hypothetical regulator 1 (YREG1), was required to mediate between
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SNAIL/SLUG transcriptional activity and the upregulation of LEF-1 expression
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following TGF- β 1/2 exposure. SNAIL/SLUG are well known transcriptional
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repressors [30–32], although there are a few studies which suggest that at least
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SNAIL can also act as a transcriptional activator [33]. In the model, we assumed
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the expression of SNAIL/SLUG was likely regulated by AP1/SP1 [34]. Thus,
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upon receiving a TGF- β 1/2 signal, the model predicted enhanced SNAIL/SLUG
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expression, consistent with experimental observations. TGF- β 1/2 stimulation
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also induces LEF-1 expression. However, literature evidence suggested that
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LEF-1 expression was not strongly dependent upon AP1/SP1 activity [35]. Thus,
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either SNAIL/SLUG are acting as inducers (contrary to substantial biochemical
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evidence) or, they are repressing the expression of an intermediate repressor.
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Given the biochemical evidence supporting SNAIL/SLUG as repressors, we
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created YREG1 a hypothetical intermediate repressor whose expression is
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downregulated by SNAIL/SLUG. The literature data therefore suggested that
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YREG1 had two transcriptional targets, LEF-1 and TGF- β 3. By adding this
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regulator, our simulations became consistent with training and literature data.
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Medici et al. suggested a similar idea where feedback between β -catenin and
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LEF-1 was likely, although this feedback had yet to be identified [7]. Low levels
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of YREG1 expression were used in all simulations to regulate the formation of
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the β -catenin-LEF-1 complex. To test the potency of YREG1, we conducted
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knockdown and over-expression simulations following the addition of TGF- β 1/2
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(Fig. S8). In the absence of YREG1, most of the population failed to
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consistently respond to TGF- β 1/2 exposure compared to the wild-type (Fig.
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S8A). Conversely, YREG1 overexpression revealed an exclusively epithelial
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phenotype following TGF- β 1/2 stimulation (Fig. S8B). Overexpression of
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YREG1 repressed LEF-1 and TGF- β 3 expression, thereby not allowing free
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 β -catenin to form the β -catenin-LEF-1 complex which promotes mesenchymal
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gene expression, or SMAD activity following from autocrine TGF- β 3 signaling.
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Likewise, the abundance of the pSmad2/4-LEF-1 complex was also reduced in
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cells overexpression YREG1, which blocked the repression of E-cadherin. Taken
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together, we found that low YREG1 expression was necessary for stabilizing
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EMT, while elevated YREG1 levels limited the extent of EMT induction.
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TGF- β 1/2 and VEGF-A exposure promotes phenotype heterogeneity through NFATc and phosphorylated Sp1

While we captured the central tendency of many of the molecular features of EMT induction following TGF- β 1/2 exposure, an often neglected but important emergent feature of developmental and pathological programs is population heterogeneity [36]. We (and others) previously hypothesized that deterministic model ensembles can interrogate population behavior, at least at a coarse grained level [20]. We tested this hypothesis by analyzing the response of the population of EMT models to extracellular cues and then comparing this response to flow cytometry studies. We used robustness coefficients to quantify the response of the individual members of the ensemble to TGF- β 1/2 stimulation. We have previously used robustness coefficients to systematically quantify response of a system to structural or operational perturbations, for example gene deletions or the addition of a growth factor or hormone [19, 20, 27, 37]. Robustness coefficients quantify shifts in molecular marker abundance resulting from molecular or environmental perturbations relative to an unperturbed control state. Robustness coefficients $\gg 1$ indicate that marker abundance increased, while robustness coefficients $\ll 1$ indicates marker abundance decreased relative to an unperturbed control. A value of ~ 1 indicates approximately no change in marker abundance following the perturbation. We calculated robustness coefficients for each member of the ensemble ($N \simeq 1100$) for two downstream phenotypic markers, Vimentin (mesenchymal) and E-cadherin (epithelial) following the addition of TGF- β 1/2 alone (Fig. 3), and VEGF-A in combination with NFATc inhibitors (Fig. 4). The absence of TGF- β 1/2 or VEGF-A stimulation was used as the baseline for the robustness calculations.

We identified model subpopulations that exhibited different behaviors following exposure to TGF- β 1/2 (Fig. 3A, labeled P1-P4). Analysis of the molecular signatures of these subpopulations suggested the abundance, localization and state of the Sp1, AP-1 and NFATc transcription factors controlled population heterogeneity. The behavior of the majority of models (>70%) was similar to subpopulation one (P1) or subpopulation two (P2) in Fig 3. These models showed the classically expected behavior, a switch from an epithelial to mesenchymal phenotype following TGF- β 1/2 exposure. Models near P1 had elevated nuclear localized phosphorylated Sp1, relative to non-induced cells (and models near P2). Elevated Sp1 activity decreased E-cadherin expression through Slug-mediated inhibition, which in turn increased Vimentin expression through TGF- β 3 autocrine signaling and the liberation of β -catenin. Near P2, Sp1 transcriptional activity was lower than P1, leading to only modestly increased Vimentin expression and E-cadherin repression following TGF- β 1/2 stimulation. Near subpopulation three (P3), reduced levels of nuclear phosphorylated AP-1, Sp1, and NFAT (resulting from the loss of ERK kinase activity) were responsible for Vimentin *repression* relative to the control. However, the most biologically interesting behavior was exhibited by subpopulation four (P4). Models near P4 had elevated Sp1 and NFAT transcriptional activity, which increased *both* Vimentin and E-cadherin

expression. Analysis of these hypothetical cells suggested they had *abnormal*
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 signaling; deregulated NFAT expression and nuclear localization promoted
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 E-cadherin expression while TGF- β 1/2 induced Sp1 action promoted Vimentin
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 expression. Analysis of the connectivity and information flow through the
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 signaling architecture suggested that Sp1 and NFAT action could be
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 manipulated *independently* by simultaneous TGF- β 1/2 and VEGF-A
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 stimulation (Fig. S1).
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To test this hypothesis, we simulated the response of the network to
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 TGF- β 1/2 and VEGF-A treatment with and without NFATc inhibitors (Fig.
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 4). As expected, stimulation with VEGF-A (50 a.u.) maintained an epithelial
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 population (Q4-43.6%), while TGF- β 1/2 (10 a.u.) exposure shifted the
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 population from an epithelial (Q4-5.5%) to a mesenchymal (Q1-45.6%)
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 phenotype (Fig. 4A and Fig. 4B). On the other hand, combined stimulation
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 with TGF- β 1/2 (10 a.u.) and VEGF-A (50 a.u.) increased both E-cadherin
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 and Vimentin expression (Q2-45.3%), resulting in a hybrid phenotype with both
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 epithelial and mesenchymal characteristics (Fig. 4C). To better understand this
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 hybrid response, we quantified the simulated protein levels for E-cadherin,
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 Vimentin, phosphorylated nuclear Sp1, nuclear NFATc1, α -smooth muscle actin
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 (α -SMA) and Slug as a function of condition (Fig. S2A-C). Vimentin expression
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 was correlated with high levels of nuclear phosphorylated Sp1, following
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 TGF- β 1/2 exposure. Conversely, elevated E-cadherin expression depended
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 upon the activity of NFAT transcription factors downstream of VEGF-A
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 stimulation. To further isolate the role of NFAT on this hybrid state, we
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 simulated the inhibition of NFAT transcriptional activity across all conditions
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 (all else being equal). NFAT inhibition in combination with VEGF-A treatment
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 blocked all E-cadherin positive sets (Fig. 4D). Likewise, TGF- β 1/2 treatment
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 in combination with NFATc inhibition also resulted in the loss of E-cadherin
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 expression (Fig. 4E). Lastly, NFATc inhibition in combination with simultaneous
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 TGF- β 1/2 and VEGF-A exposure repressed nearly all E-cadherin expression,
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 shifting nearly the entire population towards a mesenchymal phenotype (Fig.
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 4F). Taken together, high levels of nuclear localized phosphorylated Sp1
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 correlated with Vimentin expression, while NFATc transcriptional activity was
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 predicted to be critical for maintaining E-cadherin expression.
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Combined TGF- β 2 and VEGF-A exposure drives heterogeneity 290 in MCF10A and DLD1 cells

The EMT model simulations suggested the transcriptional activity of NFATc
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 and Sp1 could be independently tuned to generate a hybrid cell population with
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 both epithelial and mesenchymal characteristics. To test this hypothesis, we
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 exposed either quiescent epithelial (MCF10, (Fig. 5)) or transformed epithelial
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 cells (DLD1, (Fig. S3)) to combinations of TGF- β 1/2 and/or VEGF-A. As
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 expected, treatment with TGF- β 1/2 (10ng/ml) increased Slug and Vimentin
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 expression, while repressing E-cadherin expression both at the transcript and
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 protein levels in MCF10A (Fig. 5A-B) and DLD1 cells (Fig. S4C, Fig S3 D,E).
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 Both MCF10A (Fig. 5C) and DLD1 cells (Fig. S3E,G) transitioned from
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 quiescent cobblestone morphology to spread spindle shapes, consistent with
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EMT. As predicted, we found increased nuclear localization of phosphorylated Sp1 following TGF- β 1/2 stimulation in both MCF10A (Fig. 5B,C) and DLD1 cells (Fig. S3E,F). Consistent with model predictions, VEGF-A (50ng/ml) treatment increased the abundance of NFATc1 and E-cadherin at both the transcript and protein level in both MCF10A (Fig. 5A) and DLD1 (Fig. S3A) cells. We also found that NFATc1 nuclear localization significantly increased in both MCF10 and DLD1 treated with VEGF-A independently of the abundance of nuclear localized phosphorylated Sp1 levels (Fig. 5B,C Fig.S3C,E). Interestingly, combining VEGF-A (50ng/ml) with TGF- β 1/2 (10ng/ml) resulted in significantly elevated expression of both E-cadherin and Vimentin at the transcript and protein levels in both MCF10A and DLD1 cells (Fig 5A,B; Fig S3D,E; Fig S4C). NFATc1 expression increased, while Sp1 expression was similar to the TGF- β 1/2 case alone (Fig. 5A-B, Fig S3D,E; Fig S4C)), supporting their independent regulation. The expression of Slug, and Vimentin significantly increased, while E-cadherin levels were increased in MCF10A cells (Fig 5A) and maintained at control levels in DLD1 cells (Fig. S3D). As further predicted, nuclear co-localization of both NFATc1 and phosphorylated Sp1 were apparent in MCF10A and DLD1 cells treated with both ligands (Fig. 5B,C Fig S3E,F). Taken together, combined VEGF-A and TGF- β 1/2 treatment elicited a hybrid phenotype expressing both mesenchymal and epithelial characteristics in both MCF10A and DLD1 cells. This phenotype was driven by the transcriptional activity of two key transcription factors, Sp1 and NFATc, which could be modulated independently by TGF- β 1/2 and VEGF-A exposure.

Our robustness analysis predicted that NFATc transcriptional activity was critical to maintaining E-cadherin expression in the presence of both VEGF-A and TGF- β 1/2. We experimentally tested this hypothesis by exposing both MCF10A (Fig. 5E,F) and DLD1 cells (Fig. S4) to combinations of VEGF-A and TGF- β 1/2 in the presence or absence of VIVIT, a soluble peptide inhibitor of NFATc transcriptional activity [38]. Treatment with VEGF-A (50ng/ml) and VIVIT (10 μ M) in MCF10A cells significantly reduced E-cadherin expression compared to VEGF-A alone (Fig 5D,E). Co-treatment with VIVIT and TGF- β 1/2 did not enhance EMT capacity of MCF10A cells above that of TGF- β 1/2 alone (Fig 5A,B,E). Likewise, VIVIT in combination with both TGF- β 1/2 and VEGF-A resulted in a loss of E-cadherin gene and protein expression, while Slug and Vimentin levels remained increased (Fig. 5D,E). Quantitative flow cytometry confirmed these results in both MCF10A (Fig. 5F) and DLD1 cells (Fig. S4C). Both epithelial cell lines initially had high levels of E-cadherin expression, and low vimentin abundance (Q1-99.5%), but both MCF10A and DLD1 cells shifted from an epithelial to mesenchymal phenotype (Q1-33.4%, Q4-42.8%) following TGF- β 1/2 exposure. As expected, NFATc nuclear localization was repressed with VIVIT treatment regardless of ligand stimulation, while the abundance of nuclear phosphorylated Sp1 increased for both TGF- β 1/2 and TGF- β 1/2 + VIVIT conditions (Fig. 5D,E). Combined TGF- β 1/2 and VEGF-A increased both Vimentin and E-cadherin expression (Q1-42.1%, Q2-52.3%) compared to TGF- β 1/2 alone. Together, these results demonstrate that NFATc and phosphorylated Sp1 are critical for regulating

E-cadherin and Vimentin expression during phenotype heterogeneity in MCF10A and DLD1. 348
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Ductal branching during acini formation is dependent upon phenotype heterogeneity in MCF10A and DLD1 cells 350

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We finally employed established three-dimensional (3D) *in vitro* models of invasion, migration, compaction, and tubulogenesis [39] to determine the functional consequences of the hybrid phenotype (Fig. 6). MCF10A and DLD1 cells were aggregated via hanging drop, placed on the surface of a collagen gel, and cultured for 72 hrs under various biochemical treatments. TGF- β 1/2 stimulation significantly enhanced cell matrix invasion and matrix compaction, while in contrast VEGF-A stimulation promoted surface migration but no invasion or compaction (Fig. 6B-D). Interestingly, combined TGF- β 1/2 and VEGF-A stimulation significantly increased cell migration potential above that of VEGF-A alone while maintaining 3D matrix compaction, though with decreased magnitude compared to TGF- β 1/2 alone. Inhibition of NFATc transcriptional activity by VIVIT decreased migration following treatment with VEGF-A alone (Fig. 6B). Co-treatment of VIVIT significantly decreased migration, while complementarily increasing invasion and compaction, when MCF10A cells were stimulated with both VEGF-A and TGF- β 1/2 (Fig. 6B-D). The responses of DLD1 cells followed a similar trend to MCF10A, although the magnitudes of migration, invasion, and compaction were less. Cell circularity within 3D gels strongly and negatively correlated with both invasion and compaction regardless of treatment (Fig. 6E). Circularity refers to the morphology of the cells. In general, a quiescent epithelial cells assumes a circular morphology in culture, while an active mesenchymal cell is highly elongated. The circularity index, a common means of quantifying cell morphology, relates cell area to perimeter. A perfect circle has a circularity index equal to 1.0, while a straight line has a circularity index equal to 0.0, see Butcher et al. [40]. TGF- β 1/2 treatment alone resulted in irregular and spindle shaped morphology, while VEGF-A exposure promoted round quiescent cells (Fig. 6A). Combined VEGF-A and TGF- β 1/2 promoted morphology between these extremes. VIVIT mediated NFATc inhibition significantly reduced the circularity index, similar to TGF- β 1/2 treatment (Fig. 6F). VEGF-A treatment also induced the formation of tubular structures (acini), but the number of tubular branches relative to total acini was significantly increased upon combined TGF- β 1/2 and VEGF-A. No tubular structures were identified within the DLD1 constructs during the 7 day tubulogenesis endpoints, supporting that MCF10A and DLD1 cells have some cell-type specific EMT sensitivity despite their underlying competency for acquiring a heterogeneous phenotype. This suggests that initial EMT sensitivity of a cell influences downstream functional response from TGF- β and VEGFA stimulation. Together, these results establish that VEGF-A and TGF- β 1/2 ligand concentrations potentiate between acini and ductal branch formation in 3D culture, and are dependent upon NFATc activity.

Discussion

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In this study, we developed a family of mechanistic models describing the induction of EMT by TGF- β isoforms in the presence and absence of VEGF-A. The signaling architecture encoded in the model, which contained 995 molecular species interconnected by 1700 interactions, described the expression of 80 genes in response to growth factor stimulation. This simulation incorporates an unprecedented level of detail compared to previous models, but as a consequence created a large number of unknown model parameters. Because these parameters could not be estimated uniquely apriori, we estimated an ensemble of likely parameters using the POETs multiobjective optimization framework. The model population was trained and cross-validated to prescribe biological significance using 41 data sets generated in DLD1 colon carcinoma, MDCKII, and A375 melanoma cell lines [7]. POETs generated $> 15,000$ probable parameter sets using this data, from which we selected $N \simeq 1100$ for subsequent analysis. Analysis of this population predicted possible phenotypic modes (and their associated signaling) that cells could exhibit when stimulated with TGF- β and/or VEGF-A. The most novel hypothesis generated from the analysis was that cells could operate in a hybrid state defined by both epithelial and mesenchymal traits when stimulated simultaneously with TGF- β and VEGF-A. We tested this hypothesis in MCF10A and DLD1 cells stimulated with combinations of TGF- β and VEGF-A. As expected, in the presence of TGF- β or VEGF-A alone, MCF10A and DLD1 cells were either mesenchymal or epithelial, respectively. However, with both TGF- β and VEGF-A, MCF10A and DLD1 cells exhibited a hybrid phenotype, having both epithelial and mesenchymal characteristics. Furthermore, we found that functional traits such as tubulogenesis and ductal branching were different for cells in this hybrid phenotype. Together, this study established a predictive model of EMT induction, determined that deterministic model ensembles could predict population heterogeneity, and proved the existence of a unique hybrid phenotype resulting from the simultaneous integration of extracellular growth factor signals.

Cells routinely process a multitude of signals simultaneously, especially when coordinating developmental or pathological programs. For example, oncogenic cells integrate both mechanical and chemical cues in their local microenvironment during tumorigenesis, including cytokines VEGF and TGF- β [41]. VEGF-A mediates pathological angiogenic remodeling of tumors [42], while TGF- β can elicit both protective and oncogenic responses [43,44]. While much research has tested signaling pathways individually, far less is understood about combinatorial stimulation, such as with both VEGF-A and TGF- β . Recent *in vitro* and *in vivo* evidence has suggested that epithelial cells can exhibit heterogeneous phenotypes in addition to classically defined epithelial or mesenchymal states [45,46]. For example, expression profiling in human epithelial cancer cell lines demonstrated a spectrum of phenotypes, including some that expressed both E-cadherin and Vimentin simultaneously [47,48]. Zajchowski *et al.*, speculated that these expression profiles were somehow important for maintaining epithelial properties,

while simultaneously allowing other functional behavior such as proliferation and migration [49]. Whether and how heterogeneous phenotypes arise and participate in cancer progression, as well as their response to pharmacological inhibition are fundamental questions that should receive increased attention. In this study, we determined that a hybrid phenotype could be obtained through combined treatment with VEGF-A and TGF- β , both common factors localized in the tumor microenvironment. Furthermore, our systematic simulation-experimentation strategy identified that the transcriptional activity of Sp1 and NFATc were the critical factors controlling this phenotypic heterogeneity. Several studies have highlighted the importance of NFATc as a key transcription factor involved in cell growth, survival, invasion, angiogenesis and cancer [50]. For example, proliferation and anchorage-independent growth of pancreatic tumor cells is dependent on calcineurin and NFATc1 activity, consistent with the high levels of nuclear NFATc1 found in pancreatic tumors [51]. Likewise, our results found that VEGF-A was a potent inducer of NFATc expression, which may be required for epithelial cell migration and tubulogenesis. Although specific NFATc isoforms were not distinguished in the model, our simulations suggested that NFATc transcriptional activity was capable of maintaining epithelial traits, even during TGF- β induced EMT. Experimentally, we found that E-cadherin expression was dependent upon NFATc dephosphorylation in response to simultaneous VEGF-A and TGF- β 1/2 treatment. Thus, these results support the hypothesis that NFATc activity plays a critical role in maintaining cell-cell contacts, even during partial EMT.

Epithelial cells reproduce tissue-like organization when grown in a three-dimensional extracellular matrix (ECM) environment, and therefore are an attractive model to study morphogenic mechanisms. It is well established that MCF10A cells form structures that closely resemble acini (multi-lobed cluster of cells) in three-dimensional *in vitro* cultures [52]. It has been postulated that a cellular response reminiscent of partial EMT underlies this process, stimulating further branching and formation of acini [53]. Normally well controlled process such as tubulogenesis can be co-opted by cancer cells to break away from a primary lesion and invade through the surrounding stroma [54]. However, by retaining a transient hybrid EMT-like state, clusters of these tube-forming tumor cells can reform at a high rate after invasion, possibly explaining why invasive human carcinomas frequently appear to be cellular collections with varying degrees of gland-like differentiation [55]. In this study, we showed that our predicted hybrid phenotype generated by simultaneous treatment of epithelial cells with VEGF-A and TGF- β possessed altered migration and invasion, which enhanced tubular branching. A salient feature of this behavior, however, was the retention of cell-cell contacts that allowed cells to migrate without completely dissociating from their neighbors. Thus, our results support a mechanism in which hybrid cells can maintain some functional characteristics of epithelial cells such as cell-cell adhesion, which are normally lost in a fully differentiated mesenchymal state. The tumor microenvironment contains many soluble signals simultaneously, including VEGF and TGF- β . Thus, it is likely that some cancerous epithelial cells could exhibit hybrid EMT phenotypic states.

This may explain why fibroblastoid morphology, a classical feature of EMT, is
482 not commonly observed in human carcinomas [55]. This study focused on the
483 combinatorial effects of two very different ligand families present together in the
484 tumor environment. Additional modeling studies are required to unravel the
485 global response of epithelial cells to the full spectrum of chemical, substrate, and
486 mechanical cues. The simulation strategy presented here is readily adaptable to
487 larger species sets, with the major advantage that experimentally testable
488 hypotheses can be generated regarding how signals get integrated to produce
489 global cellular response. Furthermore, by simulating multiple ensembles of
490 parameter sets, subpopulations across a constellation of phenotypes can be
491 created and mined for common and/or divergent signaling characteristics. This
492 is a significant advantage over forced convergence to a single unique solution and
493 thereby generating a potentially non-physiological homogeneous population.
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The deterministic population of EMT models predicted heterogeneous
495 behavior that was qualitatively consistent with experimental studies. There is a
496 diversity of algorithmic approaches to estimate model parameters [56], as well as
497 many strategies to integrate model identification with experimental
498 design [57,58]. However, despite these advances, the identification of models
499 describing intracellular network behavior remains challenging. There are
500 different schools of thought to deal with this challenge. One school has focused
501 on model reduction. Data-driven approaches [15], boolean [59] or other logical
502 model formulations [16,60] are emerging paradigms that constrain model
503 complexity by the availability of the training and validation data. Other
504 techniques such as constraints based modeling, which is commonly used to
505 model metabolic networks, have also been applied to model transcriptional
506 networks, although primarily in lower eukaryotes and prokaryotes [61]. These
507 techniques (and many others, see review [62]) are certainly exciting, with many
508 interesting properties. However, we used the traditional approach of mass action
509 kinetics within an ordinary differential equation framework. The identification
510 problem for the EMT model was massively underdetermined. This is not
511 uncommon for differential equation models, especially those that are highly
512 mechanistic. Of course, we could have discarded mechanism or reduced the
513 model scope to decrease the complexity of the identification problem. However,
514 a central criticism leveled by biologists is that model simplification is often done
515 at the cost of biological reality, or done for reasons of computational
516 expediency [63]. To avoid this criticism, we systematically identified an
517 ensemble of likely models each consistent with the training data, instead of a
518 single but uncertain best fit model. Previously, we (and others) have suggested
519 that deterministic ensembles could model heterogeneous populations in
520 situations where stochastic computation was not feasible [20]. Population
521 heterogeneity using deterministic model families has previously been explored
522 for bacterial growth in batch cultures [64]. In that case, distributions were
523 generated because the model parameters varied over the ensemble, i.e., extrinsic
524 noise led to population heterogeneity. In this study, parameters controlling
525 physical interactions such as disassociation rates or the rate of assembly or
526 degradation of macromolecular machinery such as ribosomes were widely
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distributed over the ensemble. Population heterogeneity can also arise from intrinsic thermal fluctuations, which are not captured by a deterministic population of models [65]. Thus, deterministic ensembles, provide a coarse-grained or extrinsic-only ability to simulate population diversity. Despite this limitation, our prediction of phenotypic heterogeneity (and the underlying signaling events responsible for the heterogeneity) was consistent with experimental observations. This suggested that deterministic ensembles could simulate disease or developmental processes in which heterogeneity plays an important role, without having to resort to stochastic simulation.

A common criticism of ODE modeling has been the poorly characterized effect of structural and parametric uncertainty. In this study, parametric uncertainty was addressed by developing an ensemble of probable models instead of a single best-fit but uncertain model using multiobjective optimization. While computationally complex, multiobjective optimization is an important tool to address qualitative conflicts in training data that arise from experimental error or cell line artifacts [66]. On the other hand, structural uncertainty is defined as uncertainty in the biological connectivity. The EMT model connectivity was assembled from an extensive literature review. However, several potentially important signaling mechanisms were not included. First, we identified a potential gap in biological knowledge surrounding the regulation of LEF-1 expression, that was filled by the addition of the hypothetical YREG1 transcriptional repressor. The LEF-1 transcription factor is expressed in tissues that undergo EMT during embryogenesis [67, 68], and has been suggested to promote an invasive phenotype in cancer cells [8, 69]. Low levels of YREG1 were important for stabilizing the interaction between LEF-1 and β -catenin, while elevated levels inhibited EMT by downregulating LEF-1 transcriptional activity. Recent evidence has established a complex role of Amino terminal Enhancer of Split (AES) and Groucho/TLE on suppressing LEF-1 activity. AES opposes LEF-1 transcriptional activation while Groucho/TLE binds with LEF-1 for a histone deacetylase repression. In addition, β -catenin directly displaces Groucho/TLE repressors from TCF/LEF-1 in Wnt-mediated transcription activation [70, 71]. Our model agrees with this newly discovered feedback system, as YREG1 regulates LEF-1 activity leading to EMT stabilization. Secondly, we should revisit the role of GSK-3 β . GSK-3 β is an important regulator which controls the abundance of both Snail and β -catenin through the ubiquitin-proteasome pathway [72, 73]. Specifically to our model, expression of Snail increases through 72 hrs. In contrast, experimental data has shown that activity of Snail peaks at 24 hrs which may be controlled by the GSK-3 β complex [6]. Recent evidence has also suggested an essential role of NF- κ B in epithelial transformation. NF- κ B may influence Snail expression through the AKT pathway and directly stabilize Snail activity [74]. This is particularly important for integrating inflammation pathways, such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), which have been linked to EMT in pathological conditions [75]. Other pathways such as Notch have also been shown to act synergistically with TGF- β to express Slug in the developing embryo [76]. Lastly, while we have modeled classical protein signaling, we have

not considered the role of regulatory RNAs on EMT. There is growing evidence
that microRNAs (miRNAs) play a strong role in EMT, where several miRNAs,
for example miR-21 and miR-31 are strongly associated with TGF- β
exposure [77]. Addressing missing structural components like these, could
generate more insight into TGF- β signaling and its role in phenotypic
transformation.

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Materials and Methods	580
The simulation code and parameter ensemble used in this study can be downloaded from GitHub (https://github.com/jeffreyvarner/TGFb-VEGFA-Model_v1.git).	581 582 583
Signaling network connectivity	584
The EMT model described the gene expression program resulting from TGF- β and VEGF-A signaling in a prototypical epithelial cell. The TGF- β -EMT network contained 995 nodes (proteins, mRNA or genes) interconnected by 1700 interactions. The network connectivity was curated from more than 40 primary literature sources in combination with on-line databases [78, 79]. The model interactome was not specific to a single epithelial cell line. Rather, we assembled canonical pathways involved in TGF- β and VEGF-A signaling, defaulting to human connectivity when possible. Using a canonical architecture allowed us to explore general features of TGF- β induced EMT without cell line specific artifacts. On the other hand, because of the canonical architecture, we evaluated the simulation conclusions in several cell lines to test the generality of our conclusions.	585 586 587 588 589 590 591 592 593 594 595 596
Our signaling network reconstruction was based on Medici et al. who identified the pathways through which MDCKII, DLD1 colon carcinoma, and A375 melanoma cells transition towards a mesenchymal phenotype [7]. Sequential activation of MAPK and Smad pathways were initiated upon addition of TGF β 1/2. Briefly, TGF β 2 signals through the RAS-RAF-MEK-ERK pathway to up-regulate Snail and Slug expression [6]. Snail, a known repressor of junctional proteins, inhibits the expression of E-cadherin [69]. This initial repression of E-cadherin leads to a release of β -catenin from the cell membrane. Cytosolic β -catenin can then translocate to the nucleus and form transcriptional complexes with TCF-4 to drive TGF β 3 expression [7]. TGF β 3 signals to the cells interior by binding to type II receptors, which form heterodimers with type I receptors (ALK5) [80]. This activates the receptor serine/threonine kinase activity to phosphorylate and activate the receptor Smads 2/3 [81]. Phosphorylated Smads 2/3 (pSmad2/3) form heterodimers with partner Smad4 and translocate to the nucleus. pSmads complexes up-regulate other transcription factors, such as LEF-1. The pSmad2/4/LEF-1 has been shown to directly repress the E-cadherin gene [82]. LEF-1 also binds with β -catenin to upregulate mesenchymal proteins such as fibronectin [83]. The EMT gene expression program was initiated by the binding of TGF- β isoforms to TGF- β surface receptors. Binding of extracellular TGF- β 1/2 with TGF- β surface receptors I/II (TGF- β R-I/II) initiates the assembly of adapter complexes which starts the downstream signaling program. In the model, TGF- β 1/2 binds TGF- β R-I/II followed by the recruitment of activin receptor-like kinase 1 (ALK1) and TGF- β surface receptor III (TGF- β R-III) to form the activated receptor complex [80]. Alternatively, we also included activin receptor-like kinase 5 (ALK5) recruitment in combination with Endoglin and TGF- β R-III as a second (redundant) activated receptor	597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623

complex [84]. Complex assembly activates the serine/threonine kinase activity on the receptor, leading to the recruitment and phosphorylation of Smad partners [81]. Phosphorylated Smads2/3 (pSmad2/3) form heterodimers with partner Smad4 and then translocate to the nucleus where they act as both transcriptional activators and repressors. Nuclear pSmad2/3-Smad4 form transcriptional complexes with several genes in the model including lymphoid enhancer-binding factor 1 (*LEF-1*), Nuclear factor of activated T-cells, cytoplasmic 1 (*NFACT1*), and Specificity Protein 1 (*SP1*). On the other hand, nuclear pSmad2/3-Smad4 represses (in combination with the LEF-1 protein) the expression of E-cadherin (*Cdh1*) [82] and Cadherin 5, type 2 (VE-Cadherin encoded by *Cdh5*). Repression of E-cadherin expression is the central event in the transition from an epithelial to a mesenchymal phenotype [69]. However, this transition is not solely driven by transcriptional events. At the protein level, the repression of E-cadherin leads to a release of β -catenin from cell membrane. Cytosolic β -catenin then translocates to the nucleus and forms transcriptionally-active complexes with immunoglobulin transcription factor 2 (TCF-4) to drive TGF- β 3 expression [7]. Simultaneously, ERK1/2-mediated phosphorylation of the AP1 and Sp1 transcription factors can also regulate transcriptional complexes involving NFAT, Slug, and Smads. Lastly, canonical pathways for processing extracellular VEGF-A, BMP and Wnt signals, in addition to the PI3K pathway were also included in the model. Additional information about the interactions included in the model, along with the Systems Biology Markup Language (SBML) file encoding these interactions are included in the supplemental materials.

Formulation, solution and analysis of the EMT model equations

EMT was modeled using mass-action kinetics within an ordinary differential equation (ODE) framework:

$$\frac{d\mathbf{x}}{dt} = \mathbf{S} \cdot \mathbf{r}(\mathbf{x}, \mathbf{k}) \quad \mathbf{x}(t_0) = \mathbf{x}_0 \quad (1)$$

The quantity \mathbf{x} denotes the vector describing the abundance of protein, mRNA, and other species in the model (995×1). The stoichiometric matrix \mathbf{S} encodes the signaling architecture considered in the model (995×1700). Each row of \mathbf{S} describes a signaling component while each column describes a particular interaction. The (i, j) element of \mathbf{S} , denoted by σ_{ij} , describes how species i is involved with interaction j . If $\sigma_{ij} > 0$, species i is produced by interaction j . Conversely, If $\sigma_{ij} < 0$, then species i is consumed in interaction j . Lastly, if $\sigma_{ij} = 0$, then species i is not involved in interaction j . The term $\mathbf{r}(\mathbf{x}, \mathbf{k})$ denotes the vector of interactions rates (1700×1). We modeled each network interaction (gene expression, translation and biochemical transformations) using elementary rate laws where all reversible interactions were split into two irreversible steps (supplemental materials). Thus, the rate expression for interaction q was given by:

$$r_q(\mathbf{x}, k_q) = k_q \prod_{j \in \{\mathbf{R}_q\}} x_j^{-\sigma_{jq}} \quad (2)$$

The set $\{\mathbf{R}_q\}$ denotes reactants for reaction q , while σ_{jq} denotes the stoichiometric coefficient (element of the matrix \mathbf{S}) governing species j in reaction q . The quantity k_q denotes the rate constant (unknown) governing reaction q . Model equations were generated in the C-programming language using the UNIVERSAL code generator, starting from an text-based input file (supplemental materials). UNIVERSAL, an open source Objective-C/Java code generator, is available as a Google Code project (<http://code.google.com/p/universal-code-generator/>). Model equations were solved using the CVODE solver in the SUNDIALS library [85] on an Apple workstation (Apple, Cupertino, CA) as previously described [27].

Estimation of model parameters using multiobjective optimization.

The EMT model had 1756 unknown parameters (1700 kinetic constants and 56 non-zero initial conditions) which were not uniquely identifiable given the training data. Instead, we estimated a population of likely models (each consistent with the training data) using 41 data sets generated in DLD1 colon carcinoma, MDCKII, and A375 melanoma cells taken from Medici *et al.* [7]. We used the Pareto Optimal Ensemble Technique (POETs) multiobjective optimization framework in combination with leave-one-out cross-validation to estimate an ensemble of model parameters [19]. Cross-validation was used to calculate both training and prediction error during the parameter estimation procedure [86]. The 41 intracellular protein and mRNA data-sets used for identification were organized into 11 objective functions. These 11 objective functions were then partitioned, where each partition contained ten training objectives and one validation objective. The training and validation data were Western blots. Thus, all model simulations were in arbitrary units. However, POETs does allow a soft constraint on the order of magnitude of the model concentration scale. In this study, we assumed the natural model concentration scale was pmol/L. We did not place a lower bound on model states. However, based on the pmol/L natural scale, we treated all values less than 10^{-3} as zero (or no expression).

Robustness coefficients.

Robustness coefficients were calculated as shown previously [20, 27]. Robustness coefficients denoted by $\alpha(i, j, t_o, t_f)$:

$$\alpha(i, j, t_o, t_f) = \left(\int_{t_o}^{t_f} x_i(t) dt \right)^{-1} \left(\int_{t_o}^{t_f} x_i^{(j)}(t) dt \right) \quad (3)$$

quantify the response of a marker to a structural or operational perturbation to the network architecture. Here t_o and t_f denote the initial and final simulation time respectively, while i and j denote the indices for the marker and the perturbation respectively. A value of $\alpha(i, j, t_o, t_f) > 1$, indicates increased marker abundance, while $\alpha(i, j, t_o, t_f) < 1$ indicates decreased marker abundance following perturbation j . If $\alpha(i, j, t_o, t_f) \sim 1$ the j th perturbation

does not influence the abundance of marker i . Robustness coefficients were
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calculated for each member of the ensemble ($N \simeq 1100$).
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Cell culture and experimental interrogation

DLD1 colon carcinoma, MCF10A, and HUVEC were acquired from the
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American Tissue Culture Collection (Manassas, VA). Cells were grown in
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culture with RPMI 1640 medium with 10% fetal bovine serum and 1%
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penicillin/streptomycin for DLD1, EBM-2 supplemented with EGM-2, 5% fetal
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bovine serum, and 1% penicillin/streptomycin for HUVEC, or MGEM 2
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supplemented with insulin, bovine pituitary extract, cholera toxin, hEGF,
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hydrocortisone, 5% horse serum, and 1% penicillin/streptomycin for MCF10A.
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Cells were serum starved for 24 hours and removed from all experimental
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conditions. Recombinant VEGFA165 was also removed from culture medium
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prior to experimentation. Recombinant human TGF- β 2 (R & D Systems,
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Minneapolis, MN) was added to the culture medium at a concentration of 10
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ng/ml and recombinant VEGFA165 at a concentration of (5ng/ml, 50ng/ml) for
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all relative experiments. NFAT inhibitor (VIVIT peptide) (EMD Biosciences,
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Darmstadt, Germany), was added to the culture medium at a concentration of
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 $10\mu\text{M}$ for all relative experiments. Cells were passaged 1:3 or 1:4 every 3-6 d and
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used between passages 4 and 8.
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RT-PCR

RNA extractions were performed using a Qiagen total RNA purification kit
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(Qiagen, Valencia, CA) and RNA was reverse transcribed to cDNA using the
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SuperScript III RT-PCR kit with oligo(dT) primer (Invitrogen). Sufficient
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quality RNA was determined by an absorbance ratio A260/A280 of 1.8-2.1,
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while the quantity of RNA was determined by measuring the absorbance at
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260nm (A260). Real-time PCR experiments were conducted using the SYBR
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Green PCR system (Biorad, Hercules, CA) on a Biorad CFX96 cycler, with 40
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cycles per sample. Cycling temperatures were as follows: denaturing, 95C;
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annealing, 60C; and extension, 70C. Primers were designed to detect GAPDH,
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E-cadherin, vimentin, Slug, Sp1, and NFATc1 in cDNA clones: Sp1 (F-TTG
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AAA AAG GAG TTG GTG GC, R-TGC TGG TTC TGT AAG TTG GG,
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Accession NG030361.1), NFATc1 (F-GCA TCA CAG GGA AGA CCG TGT C,
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R-GAA GTT CAA TGT CGG AGT TTC TGA G, Accession NG029226.1).
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GAPDH, E-cadherin, vimentin, and Slug primers were taken from previously
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published literature [7].
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Antibody Staining

Samples were fixed in 4% PFA overnight at 4C. Samples were then washed for
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15 minutes on a rocker 3 times with PBS, permeabilized with 0.2% Triton-X 100
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(VWR International, Radnor, PA) for 10 minutes, and washed another 3 times
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with PBS. Samples were incubated overnight at 4C in a 1% BSA (Rockland
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Immunochemicals, Inc., Gilbertsville, PA) blocking solution followed by another
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4C overnight incubation with either rabbit anti-human E-cadherin 1:100
 (Abcam, ab53033), mouse anti-human phospho-Sp1 1:100 (Abcam, ab37707),
 mouse anti-human vimentin 1:100 (Invitrogen, V9), and rabbit anti-human
 NFATc1 (Santa Cruz, sc-7294) 1:100. After 3 washes for 15 minutes with PBS,
 samples were exposed to Alexa Fluor 488 or 568 conjugated (Invitrogen), species
 specific secondary antibodies at 1:100 in 1% BSA for 2 hours at room
 temperature. Three more washes with PBS for 15 minutes were followed by
 incubation with either DRAQ5 far red nuclear stain (Enzo Life Sciences,
 Plymouth Meeting, PA) at 1:1000.

FACS

Flow cytometry for E-cadherin 1:100 (Abcam) and vimentin 1:100 expressing
 cells was performed. Briefly, cells were trypsinized, fixed with 4% PFA for 10
 min and then preserved in 50% methanol/PBS. Cells were kept in the -20C until
 antibody staining was preformed. Samples were divided into multiple aliquots in
 order to stain the proteins separately and compensate for secondary antibody
 non-specific binding. Cells were incubated for 24 hrs at 4 C in primary antibody
 diluted in either PBS (extracellular) or 0.2% saponin-PBS (intracellular). Cells
 were then washed 3 times with PBS and incubated with appropriate secondary
 antibodies and imaged using a Coulter Epics XL-MCL Flow Cytometer
 (Coulter). All samples were compensated using appropriate background
 subtraction and all samples were normalized using 7500 cells per flow condition.

Three-Dimensional Culture and Tubulogenesis Assays

For invasion/migration assays, cells were resuspended in culture media, and
 allowed to aggregate overnight in hanging drop culture (20 μ L; 20,000 cells). The
 spherical aggregates were placed on the surface of neutralized type I collagen
 hydrogels (1.5mg/mL) and allowed to adhere for 2 hrs before adding treatments.
 Cultures were maintained for 72 hrs, after which they were fixed in 4% PFA and
 slowly rehydrated using PBS. For compaction assays, cells were pelleted via
 centrifugation and resuspended within a neutralized collagen hydrogel
 (1.5mg/mL) solution at a density of 400,000 cells/mL. 250 μ L of gel was
 inoculated into culture wells, which solidified after 60min. Treatments were then
 added within 800 μ L of the culture medium without serum. Gels were liberated
 from the surfaces of the culture wells the next day and cultured free floating for
 an additional 3-7 days, exchanging serum free media with appropriate factors
 every 48 hrs.

Tubulogenesis was defined as a typical nonmalignant acini structure. This
 includes a polarized epithelial cell, hollow lumen, and the basal sides of the cell
 are surrounded by ECM proteins (Fig. 6A, Controls or VEGF treated).
 Previous work has shown that change in the morphological characteristics of
 nontumorigenic MCF10A epithelial acini occur over time and exploiting them to
 growth in 3D culture can be quantified. For example, using image segmentation,
 Chang et al. [87] examined the elongation of the MCF10A acini at 6, 12, and 96
 hours after a particular treatment. Polizzotti et al. [88] also suggested a

computational method to quantify acini structure based on morphological characteristics in nonmalignant, noninvasive, and invasive conditions. Adapted from these approaches, we first fluorescently labeled our cultures and captured the acini structures by 3D confocal microscopy. Next individual acini structures in the images were segmented by imageJ and labeled. We then extracted the number of ductal branches. Ductal branching was defined as any elongated cell cluster extending away from the total acini structure, which was manually segmented and counted using ImageJ. A total of 5 images for each condition were used, and approximately 12 acini were analyzed in each image. Total branching was normalized to the amount of acini present, and provides an overall general assessment to the extent of acini remodeling.

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Statistics

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Results are expressed as mean \pm standard error, $n \geq 6$. Data was analyzed with the GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA) and SAS (Statistical Analysis Software, Cary, NC). A one-way ANOVA with Tukey's post hoc was used to compare differences between means and data was transformed when necessary to obtain equal sample variances. Differences between means were considered significant at $p < 0.05$.

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Figure 1. Model connectivity recreates the core architecture during EMT. The EMT network contains 995 nodes (proteins, mRNA, and genes) interconnected by 1700 interactions. Central to EMT induction, activation of the MAPK cascade occurs through TGF- β 1/2 binding which activates the AP-1/Sp1 transcriptional axis. AP-1/Sp1 drives an autocrine response of TGF- β 3, which activates the Smad cascade, leading to phenotypic change. Conversely, VEGF-A binding promotes an epithelial phenotype through NFAT activation. Other important signaling pathways not shown but also incorporated include the BMP, Wnt, and PI3K pathways. The complete list of molecular interactions that comprise the model is given in the supplement.

Figure 2. Training and validation simulations. The population of EMT models qualitatively captured TGF β -induced EMT signaling. (A-I) The population was generated using POETs and trained using 11 different objective functions (41 data sets) taken from Medici *et al.* [7]. The model captured the simulated experiments for 78% of the cases. (J-L) The model populations were also compared against untrained temporal data to measure the effectiveness as a pure prediction. The high predictability can be contributed to the leave-one-out cross validation scheme, objective functions with overlapping data, and multi-objective optimization algorithm.

Figure 3. Simulated TGF β 1/2 exposure promoted phenotype heterogeneity. Robustness coefficients were used to quantify the effect of perturbations after steady state across the population of data sets. Coefficients with values > 1 (< 1) indicated a marker increased (decreased) compared to a base state, while a value of 1 indicated approximately no change following a perturbation. E-cadherin and Vimentin robustness coefficients were used as phenotypic markers. (A) TGF β 1/2 perturbation, we isolated 4 distinct parameter sets known to have phenotypically different behaviors. (B) Each region has been represented by a small signaling network. From our analysis, we determined that the differences were a function of downstream transcription factors (phosphorylated-Sp1, and NFAT) within the TGF β 2 and VEGFA pathway, respectively. (C) We hypothesized that elevated phosphorylated-Sp1 and NFAT levels could drive phenotype heterogeneity through simultaneous TGF β 2 and VEGFA treatment, similar to region three (R3).

Figure 4. Simulated response to TGF β 1/2 and VEGF-A exposure with and without axis specific inhibitors. Robustness coefficients were used to quantify the shift in population at 48 hrs. (A-C) VEGF-A (50 a.u.) treatment resulted in a population with enhanced epithelial (Q4) properties. This was contrary to the addition of TGF β 2 (10 a.u.), which shifted the population towards a mesenchymal phenotype (Q1). Interestingly, the combined effects of TGF β 2 and VEGFA was found to increase both ecadherin and vimentin levels, creating a heterogeneous population (Q2). (D-F) To isolate the effect of NFAT, we inhibited NFAT de-phosphorylation in combination with VEGFA. This negated the increase in ecadherin expression and shifted the population towards a mesenchymal phenotype (Q1,Q3). Likewise, combining NFAT inhibition with TGF β mitigated all ecadherin expression (Q2) confirming their importance for population heterogeneity. Lastly, combination of TGF β 2, VEGFA, and NFAT inhibition nearly mitigated all effects of VEGFA, shifting the heterogeneous population (Q2) towards a mesenchymal phenotype (Q1). In whole, high levels of phosphorylated-Sp1 correlated with vimentin expression, while NFAT was responsible for maintaining E-cadherin expression, although neither were mutually exclusive.

Figure 5. Simultaneous TGF- β 1/2 and VEGF-A treatment induced phenotype heterogeneity and is dependent upon NFAT activity *in-vitro*. (A) In MCF10A, treatment with (10ng/ml) TGF β 2 increased Slug and vimentin, while ecadherin expression was inhibited at both the gene and protein level at 48 hrs. Conversely, VEGFA alone increased both NFATc1 and ecadherin gene expression. Simultaneous TGF β 2 (10ng/ml) and VEGFA (50ng/ml) treatment increased Slug, NFATc1, and vimentin expression, while also increasing ecadherin levels via qPCR. (B-C) Immunofluorescence confirmed these results and nuclear co-localization of both phospho-Sp1 and NFAT were found dependent upon TGF β 2 and VEGFA, respectively. (D) To isolate the effect of NFAT, treatment of VEGFA (50ng/ml) and VIVIT (10 μ M) reduced ecadherin expression at 48hrs (control-dashed line). Similarly, combined TGF β 2, VEGFA and VIVIT treatment increased Slug and vimentin expression, while inhibiting ecadherin levels via qPCR. (E) These findings were confirmed via immunofluorescence as the VIVIT peptide inhibited ecadherin and nuclear localization of NFATc1 in all three cases. (F) Quantitative flow cytometry also confirmed this trend. Similar experiments in DLD1 followed a similar trend (supplement). Magnification, 40x. Scale bars: 50 μ m. C=Control, T=TGF β 2 , V=VEGFA, VI= NFAT inhibitor (VIVIT). Asterisks signify statistical differences from each other according to a one-way ANOVA with Tukey's post hoc ($p < 0.05$).

Figure 6. Ductal branching is dependent upon phenotype heterogeneity within MCF10A in 3-D culture. MCF10A and DLD1 were formed into spheroids overnight and explanted to a collagen gel for 72 hrs. For compaction and tubular assays, cells were embedded into collagen gels for 72 hrs, and the extent of tubulogenesis was measured at 7 days. (A-D) Within MCF10A, TGF β 2 (10ng/ml) enhanced invasion and contractile properties while, VEGFA (50ng/ml) promoted increased migration. TGF β 2 with VEGFA significantly increased migration, while limiting with compaction. VIVIT (10 μ M) in combination with VEGFA and TGF β 2 decreased migration and compaction, while increasing invasion. (D) Likewise, cell morphology (circularity index) correlated with both invasion and compaction in MCF10A. (E-F) The size of tubular structures (acini) also increased significantly upon addition of VEGFA, while the number of ductal branches was most significant upon simultaneous TGF β 2 and VEGFA treatment (Red-Ecadherin, Green-Factin, Blue-Nuclear). DLD1 cells followed a similar trend, although the degree of migration, invasion, and compaction was less significant. In addition, no tubular structures were identified during the 7 day tubulogenesis endpoints. Scale bars: 500 μ m, 1000 μ m, 250 μ m, and 80 μ m, respectively. C=Control, T=TGF β 2 , V=VEGFA, VI= NFAT inhibitor (VIVIT). Asterisks signify statistical differences from each other according to a one-way ANOVA with Tukey's post hoc ($p < 0.05$). Boxes in the left-most panel identify regions identified by arrows that were then imaged in greater zoom in the panel immediately below. The box diagram was not repeated for arrows in the other panels for clarity, but the same method was applied.

Supplemental Materials and Methods

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EMT model network architecture.

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TGF β is a major inducer of EMT in development, fibrosis, and carcinogenesis with different isoforms mediating various effects depending on specific cellular context [89]. TGF β 1 was first described as an inducer of EMT in normal mammary epithelial cells [90] and has since been shown to mediate EMT in vitro in a number of different epithelial cells, including renal proximal tubular, DLD1 colon carcinoma, and most recently alveolar epithelial cells [91–94]. TGF β signaling occurs through the Smad pathway in which signals are transduced by transmembrane serine/threonine kinase type I (ALK5) and type II (TGF β RII) receptors. To increase ligand affinity, betaglycan (TGF β RIII) can also interact with TGF β RI,II [84]. Upon TGF β stimulation, the receptors are internalized into early endosomes where Smad anchor for receptor activation (SARA) modulates formation of complexes with (R-Smad) Smad2 or Smad3. Smad2 and Smad3 are then phosphorylated at serine residues by the type I receptor [81]. Phosphorylation induces their association with (Co-Smad) Smad4 and translocation to the nucleus where they interact with other transcription factors to regulate the transcription of TGF β responsive genes, including alpha-smooth muscle actin, collagen1A2, vimentin, fibronectin, and plasminogen activator inhibitor-1 (PAI-1) by interacting with Smad-binding elements [80,95]. To regulate TGF β signaling, Smurf2 (a ubiquitin E3 ligase) can become activated to mediate proteasome dependent degradation of Smad2 or bind with Smad 7 to target TGF β receptor for degradation [96,97].

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Cell Type Dependency

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Interestingly, differential roles for Smad2 and Smad3 in TGF β induced EMT have been demonstrated. For example, using primary cells from mice with hepatocyte-specific double knockout of Smad2 and Smad3, it was demonstrated that Smad3 but not Smad2 was required for a key morphological changes and induction of EMT [98]. In contrast, using human proximal tubular epithelial cells, increased colony growth factor and decreased E-cadherin were Smad3 dependent, increased MMP-2 was Smad2 dependent, while alpha-smooth muscle actin was dependent on both [99]. Furthermore, Smad2 signaling has been demonstrated in cancerous lung epithelial A549 cells [93]. Inhibition of Smad3 signaling decreases the metastatic potential of xenografted breast cancer cell lines [100,101]. Together, these results suggest that the precise Smad pathway activated may depend on the particular cellular context. Regardless, a recent transcriptomic analysis of TGF β induced EMT in normal mouse and human epithelial cells using a dominant negative approach demonstrated that Smad signaling was critical for regulation of all tested target genes [102].

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Smad-Independent EMT Induction Pathways.

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Although less well established than the Smad-dependent pathways in the induction of EMT, there is substantial evidence for TGF β activation of

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Smad-independent signaling in some aspects of this process. These include RhoA, MAPK, PI3 kinase, and Notch signaling pathway, which have mostly been studied in-vitro. For example, the small GTPase RhoA is involved in TGF β induced EMT in a number of cell types including NMuMG mammary epithelial cells and mink lung epithelial (Mv1Lu) cells particularly in the regulation of cytoskeletal and adherens junction rearrangement. In addition to its role in cytoskeletal remodeling, Rho has been shown to activate the alpha-smooth muscle promoter during EMT in kidney proximal tubular (LLCPK1) cells [103]. TGF β has been shown to elevate ERK activity in culture models of EMT (human keratinocytes, NMuMG mammary epithelial cells, and mouse cortical tubule epithelial cells) [104–106]. This ERK activity was required for disassembly of adherens junctions and induction of cell motility [105]. Inhibition of ERK blocked these key morphological changes of EMT in mammary gland epithelial cells [107]. Recent studies in MDCKII and DLD1 colon carcinoma cells revealed that TGF β 3 can signal through PI3 kinase. Upon activation, PI3 kinase activates molecules such as ILK and AKT, which can phosphorylate and inactivate GSK3 β , a protein that targets both Snail and β -catenin for degradation through the ubiquitin proteasome pathway [7]. Lastly, there is increasing evidence for a role of Notch pathways in regulating EMT, particularly during development [89]. In cardiac cushion EMT, it was found that that Slug is directly up-regulated by Notch in endothelial cells repression of the vascular endothelial cadherin promoter and for promoting migration of transformed endothelial cells. In whole, the distinction between Smad-dependent and Smad-independent mechanisms remains difficult to interpret due to the significant cross talk between these pathways. In most cases, stimulation of these cooperative pathways provides the context for induction and specification of EMT within a particular tissue/cell type, with Smads representing the dominant pathway, which in some instances may be necessary but not sufficient for induction of full EMT [4].

Master Transcription Regulators

Master transcription factors of EMT are thought to be regulated by Snail1 (SNAI1) and Snail2 (SNAI2) (previously known as Snail and Slug, respectively). These transcription factors are zinc finger proteins that function as repressors of E-cadherin transcription in cultured epithelial cells [69], repression of E-cadherin leads to dissolution of adherens junctions. Snail and Slug can be activated by TGF β via both Smad-dependent and -independent pathways in a cell type dependent fashion in cultured cells [108]. Differential expression of Snail and Slug is observed in TGF β induced EMT in keratinocytes, renal proximal tubular, and mammary epithelial cells, suggesting that they are regulated in a cell specific. Interestingly, key regulatory units of Snail and Slug appear to be regulated by upstream transcription factors AP1 and SP1, respectively. Peinado et al. clearly established a necessary role of Snail in mediating TGF β 1 induced EMT in MDCK cells. They demonstrated that the Ras-Raf-MEK-ERK-AP1 signaling pathway could up-regulate synthesis of the E-cadherin repressor molecule Snail (directly binds to Snail promoter) [109]. Likewise, TGF β 1

induced the expression of Slug in both lens and other epithelial cells in vitro. The Sp1 binding site in the Slug promoter is largely responsible for TGF β 1 induced Slug expression and upstream of MAPK signaling. In addition, the TGF β 1 mediated repression of E-cadherin was significantly inhibited by Slug siRNA [110].

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ERK/MAPK Crosstalk

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The ERK-MAPK pathway has been shown to modify TGF β signaling at multiple levels. One way is that the activated Ras pathway inhibits the TGF β induced nuclear accumulation of Smad2/3, as seen in epithelial cells [111]. ERK kinases have been shown to phosphorylate Smad2 and Smad3 at specific sites in the region linking the MH1 and MH2 domains. These sites are separate from the TGF β receptor phosphorylation sites which activate Smad nuclear translocation [112]. The effect of interaction between ERKs and Smads is the subject of some controversy, with data suggesting that such an interaction either enhances or inhibits downstream events. A hyperactive Ras pathway has been shown to effectively counteract the antiproliferative activity of TGF β through attenuation of Smad accumulation in the nucleus [113]. In contrast, Ras signals strongly cooperated with Smads for invasion of human carcinoma cells [114]. A second way is through transcriptional regulation. Recently, it was found that Sp1, via transcriptional induction of Vimentin, cooperates with activated Smad complexes in mesenchymal transition and migration of pancreatic cancer cells upon TGF β stimulation [115]. Likewise, binding sites at the SP1/Smad3 complex was found to regulate the betaglycan receptors [116]. Taken together, multiple levels of cross-talk (both positive and negative) exist within MAPK and TGF β , and may also be cell type dependent.

Analysis of the signal flow through the EMT architecture.

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To investigate temporal shifts for key species dominating the EMT response, we calculated the scaled flux through the signaling architecture (Fig. S1). Three modes of operation were simulated to identify distinct behavioral differences: (a) no TGF- β 2 (-TGF- β 1/2), (b) TGF- β 1/2 stimulation (+TGF- β 2), and (c) TGF- β 1/2 stimulation while blocking the autocrine response of TGF- β 3 (+TGF- β 1/2 + TGF- β 3Ab). Following TGF- β 1/2 stimulation, AP1/SP1 rapidly stimulates Snail/Slug expression (Fig. S1A) within 1 hr. During this phase, Snail/Slug repress E-cadherin expression, which in turn reduces the interaction between E-cadherin and β -catenin, and the accumulation of free cytosolic β -catenin ensues. Conversely, in the presence of a TGF- β 3 neutralizing antibody, membrane bound β -catenin increased after 10 hrs, while low levels of free cytosolic β -catenin accumulated between 1-5 hrs (Fig. S2A,B). Accumulation of free β -catenin is critical for complexing with TCF4 to rapidly produce the autocrine response of TGF- β 3 within 1-10 hrs. Following TGF- β 3 expression, formation and spatial relocation of the Smad complexes occurs in a time dependent manner. Smad2 is phosphorylated within 1 hr and nuclear localization of the pSmad2/4 complex dramatically increases after 10 hours (Fig.

S1C). This timely formation is critical for completion of the EMT process. At 1300
steady state, the E-cadherin complex was maintained at a basal epithelial level 1301
with no expression of Vimentin (Fig. S1D). During TGF- β 1/2 stimulation, both 1302
MAPK and Smad act synergistically to repress the E-cadherin complex within 1 1303
hr. This is followed by increase of Vimentin at ~ 1hr, while exponentially 1304
increasing around 10 hrs. Both species elevate within 1 hr, however the complex 1305
does not significantly form until ~ 10 hrs. When blocking TGF- β 3, Snail/Slug 1306
downregulates the E-cadherin complex between 5-10 hrs, but cannot complete 1307
the transformation due to the loss of Smad2 phosphorylation (Fig. S1D). 1308

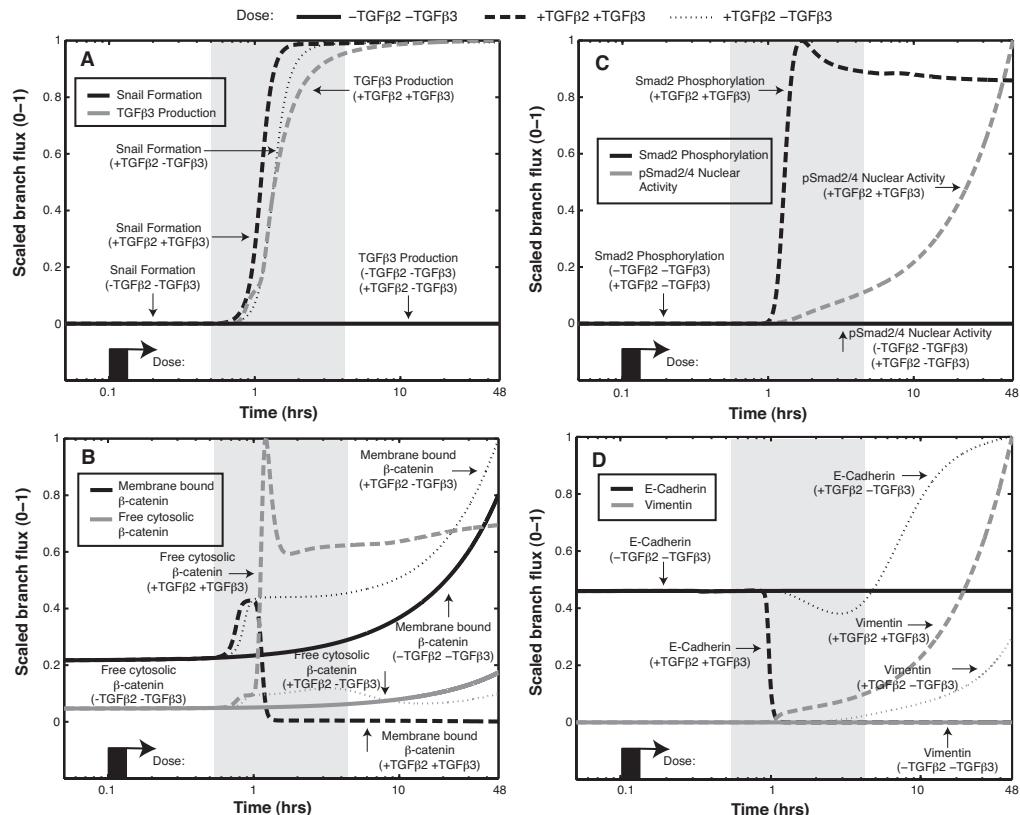


Figure S1. Signal flow analysis of key species at steady state, TGF- β 1/2 stimulation, and blocking the TGF- β 3 autocrine response. (A) The MAPK cascade is directly responsible for rapid expression of Snail and downstream TGF- β 3 formation (1 hrs). (B) TGF- β 1/2 reduces β -catenin, allowing rapid free-cytosolic β -catenin to accumulate (1hr). Blocking TGF β 3 increases membrane bound β -catenin (10hr). (C) TGF- β 3 activates the Smad cascade. Nuclear localization of the pSmad2/4 complex (10 hrs) is dependent upon both the phosphorylation of Smad2 (1 hrs) and complexing with Smad4 (5 hrs). (D) TGF- β 1/2 rapidly reduces the E-cadherin complex, while upregulating Vimentin (5-10 hrs). Blocking TGF β 3 increases E-cadherin (10 hrs) and Vimentin is significantly reduced.

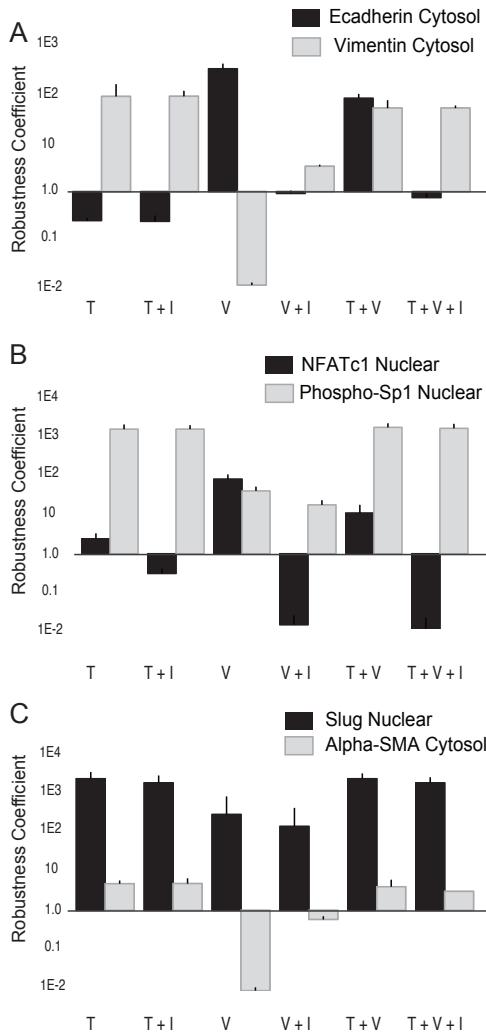


Figure S2. Robustness analysis for key molecular species at $t = 48$ hrs for combinations of TGF- $\beta 1/2$, VEGF-A and NFATc1 inhibitors. Robustness coefficients for the indicated species were calculated for $N \sim 1100$ ensemble members for 48 hrs following the addition of TGF- $\beta 1/2$ (T), TGF- $\beta 1/2$ + NFATc1 inhibitor (T + I), VEGF-A (V), VEGF-A + NFATc1 inhibitor (V + I) and TGF- $\beta 1/2$ + VEGF-A (T+V) + NFATc1 inhibitor (T + V + I). (A) Robustness coefficients for E-cadherin and Vimentin as a function of condition. (B) Robustness coefficients for nuclear localized phosphorylated Sp1 and NFATc1 as a function of condition. (C) Robustness coefficients for nuclear localized Slug and α -smooth muscle actin (α -SMA) as a function of condition. In each case the error bars denote one-standard deviation of robustness coefficient calculated over the model ensemble. C=Control, T=TGF $\beta 2$, V=VEGFA, VI= NFAT inhibitor (VIVIT).

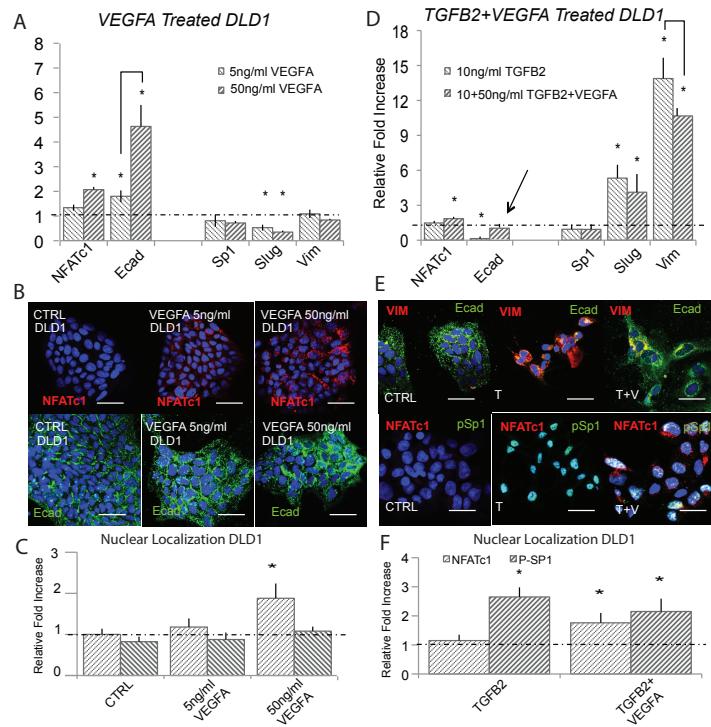


Figure S3. VEGF-A attenuates TGF- β 1/2 to induce phenotype heterogeneity in DLD1. (A) In DLD1, we found that 5ng/ml of VEGFA increased NFATc1 and E-cadherin gene expression via qPCR and 50ng/ml potentiated this effect at 48 hrs. (B - C) These findings were confirmed at the protein level via immunofluorescence, as ecadherin levels and nuclear localization of NFATc1 increased. (D) Treatment with (10ng/ml) TGF β 2 resulted in mesenchymal transformation as measured via qPCR against target genes Slug, ecadherin, vimentin, Sp1, and NFATc1. (E - F) Immunofluorescence and nuclear localization revealed a strong presence of phospho-Sp1. (G) Combination of VEGFA (50ng/ml) and TGF β 2 (10ng/ml) treatment resulted in increased Slug, NFATc1, and vimentin expression, while also increasing ecadherin levels compared to control. (H) Immunofluorescence confirmed these results, as both ecadherin and vimentin levels were elevated. (I) A significant increase in nuclear localization of both NFATc1 and phospho-Sp1 were also found. Magnification, 40x. Scale bars: 50 μ m. C=Control, T=TGF β 2 , V=VEGFA, VI=NFMAT inhibitor (VIVIT). Asterisks signify statistical differences from each other according to a one-way ANOVA with Tukey's post hoc ($p < 0.05$).

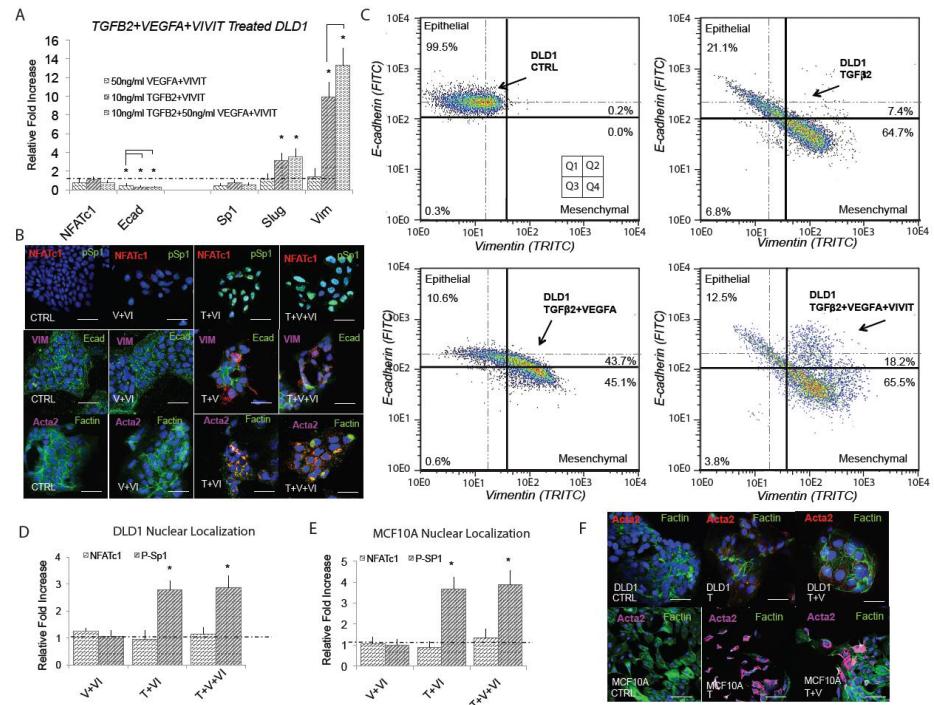


Figure S4. E-cadherin expression is dependent upon NFAT activity in DLD1. (A) Treatment with VEGFA (50ng/ml) and NFAT inhibitory peptide VIVIT ($10\mu M$) resulted in significantly reduced ecadherin expression (qRT-PCR at 48hrs). Addition of TGF β 2 (10ng/ml) and VIVIT resulted in increased Slug and vimentin expression, while inhibiting ecadherin levels. Combined TGF β 2, VEGFA, and VIVIT treatment resulted in target genes Slug and vimentin expression increased, while inhibiting ecadherin levels. No change in Sp1 or NFATc1 expression was found. (B) These findings were confirmed via immunofluorescence as the VIVIT inhibitors was shown to inhibit ecadherin levels in all three cases. We also found no change in gene or nuclear localization of NFATc1 in all three cases, while phospho-Sp1 was found to increase in both TGF β conditions. (C) Quantitative flow cytometry also confirmed this trend. (D,E) TGF β 2, VEGFA and VIVIT treatment in DLD1 and MCF10A resulted in no change of Sp1 expression or NFATc1 expression. (F) Likewise, no change in nuclear localization of NFAT in all three cases, however phospho-Sp1 was found to increase in both TGF β conditions. Magnification, 40x. Scale bars: $50\mu m$. C=Control, T=TGF β 2 , V=VEGFA, VI= NFAT inhibitor (VIVIT). Asterisks signify statistical differences from each other according to a one-way ANOVA with Tukey's post hoc ($p < 0.05$).

Estimation and cross-validation of EMT model parameters.

We used the Pareto Optimal Ensemble Technique (POETs) multiobjective optimization framework in combination with leave-one-out cross-validation to estimate an ensemble of TGF β /EMT models. Cross-validation was used to calculate both training and prediction error during the parameter estimation procedure [86]. The 41 intracellular protein and mRNA data-sets used for identification were organized into 11 objective functions. These 11 objective functions were then partitioned, where each partition contained ten training objectives and one validation objective. POETs integrates standard search strategies e.g., Simulated Annealing (SA) or Pattern Search (PS) with a Pareto-rank fitness assignment [19]. Denote a candidate parameter set at iteration $i + 1$ as \mathbf{k}_{i+1} . The squared error for \mathbf{k}_{i+1} for training set j was defined as:

$$E_j(\mathbf{k}) = \sum_{i=1}^{\mathcal{T}_j} \left(\hat{\mathcal{M}}_{ij} - \hat{y}_{ij}(\mathbf{k}) \right)^2 \quad (\text{S1})$$

The symbol $\hat{\mathcal{M}}_{ij}$ denotes scaled experimental observations (from training set j) while \hat{y}_{ij} denotes the scaled simulation output (from training set j). The quantity i denotes the sampled time-index and \mathcal{T}_j denotes the number of time points for experiment j . In this study, the experimental data used for model training was typically the band intensity from Western or Northern blots. Band intensity was estimated using the ImageJ software package [117]. The scaled measurement for species x at time $i = \{t_1, t_2, \dots, t_n\}$ in condition j is given by:

$$\hat{\mathcal{M}}_{ij} = \frac{\mathcal{M}_{ij} - \min_i \mathcal{M}_{ij}}{\max_i \mathcal{M}_{ij} - \min_i \mathcal{M}_{ij}} \quad (\text{S2})$$

Under this scaling, the lowest intensity band equaled zero while the highest intensity band equaled one. A similar scaling was defined for the simulation output. By doing this scaling, we trained the model on the relative change in blot intensity, over conditions or time (depending upon the experiment). Thus, when using multiple data sets (possibly from different sources) that were qualitatively similar but quantitatively different e.g., slightly different blot intensities over time or condition, we captured the underlying trends in the scaled data. Additionally, we applied a universal convention of identifying the no expression case as protein value below $<10^{-3}$. This is similar to previously published models from our lab. In our studies, Figure 2 identifies experimental data extracted from published Western blots and our simulation results. It is clear from these that 1) the training data included a variety of Western blot data treatments that were effectively zero, and 2) our simulations matched the training data virtually perfectly over time and across multiple biological species. These results validate the power of our simulation scheme, which necessarily includes numerical interpretations of zero. In our Supplemental Figure S9, we present the raw concentrations of our simulations (previously validated from the training data) over hundreds of parameter ensembles, with no “zero” threshold applied. While these values do not have a threshold, we only interpret values below 10^{-3} equivalent to zero. Likewise, our robustness coefficients (Figure 3)

O#	Species (cytosolic protein)	Cell Type	Training	Prediction	Random	Source
O1	LEF1	DLD1 CC, MDCKII, A375 MC	0.73 ± 0.22	0.66 ± 0.18	0.87 ± 0.02	Medici et al., 2008
O2	Vimenin	DLD1 CC, MDCKII, A375 MC	0.96 ± 0.11	1.00 ± 0.15	0.23 ± 0.04	"
O3	TGF beta3	DLD1 CC, MDCKII, A375 MC	1.00 ± 0.13	0.77 ± 0.22	0.61 ± 0.04	"
O4	E-Caderin	DLD1 CC, MDCKII, A375 MC	0.54 ± 0.16	0.00 ± 0.00	0.15 ± 0.03	"
O5	beta-catenin	DLD1 CC, MDCKII, A375 MC	0.99 ± 0.25	0.00 ± 0.00	0.00 ± 0.00	"
O6	TGFBeta3	DLD1 CC, MDCKII, A375 MC	0.89 ± 0.14	0.71 ± 0.12	0.58 ± 0.04	"
O7	Snail	DLD1 CC, MDCKII, A375 MC	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	"
O8	LEF1	DLD1 CC, MDCKII, A375 MC	1.00 ± 0.14	1.00 ± 0.25	0.61 ± 0.05	"
O9	E-Caderin	DLD1 CC, MDCKII, A375 MC	0.96 ± 0.20	0.86 ± 0.19	0.00 ± 0.00	"
O10	Slug	DLD1 CC, MDCKII, A375 MC	1.00 ± 0.20	1.00 ± 0.12	0.00 ± 0.00	"
O11	LEF1	DLD1 CC, MDCKII, A375 MC	1.00 ± 0.15	1.00 ± 0.25	0.86 ± 0.03	"
P#	Species		Simulated	Experimental	Random	Source
P1	E-caderin (mRNA)	MDCKII	0.10 ± 0.01	0.13 ± 0.02	0.27 ± 0.03	Medici et al., 2006
P2	pSmad2	MDCKII	0.58 ± 0.12	0.80 ± 0.04	0.13 ± 0.03	"
P3	LEF1 (mRNA)	MDCKII	0.76 ± 0.05	0.65 ± 0.04	0.18 ± 0.03	"

Figure S5. Training and prediction values for the 11 TGF- β objective functions versus a random parameter control.

(which represent the ratio of integrated areas of the treatment effect over the baseline effect) identify no difference in model output for vimentin (or e-cadherin for that matter) less than 10^{-3} , confirming this interpretation.

We computed the Pareto rank of \mathbf{k}_{i+1} by comparing the simulation error at iteration $i + 1$ against an archive of accepted parameter sets \mathbf{K}_i . We used the Fonseca and Fleming ranking scheme [118] to estimate the number of parameter sets in the archive that dominate \mathbf{k}_{i+1} . Parameter sets with increasing rank were progressively further away from the optimal trade-off surface. The parameter set \mathbf{k}_{i+1} was accepted or rejected by POETs with probability $\mathcal{P}(\mathbf{k}_{i+1})$:

$$\mathcal{P}(\mathbf{k}_{i+1}) \equiv \exp \{-rank(\mathbf{k}_{i+1} | \mathbf{K}_i) / T\} \quad (\text{S3})$$

where T is the annealing temperature and $rank(\mathbf{k}_{i+1} | \mathbf{K}_i)$ denotes the Pareto rank for \mathbf{k}_{i+1} . The annealing temperature was discretized into 10 quanta between T_o and T_f and adjusted according to the schedule $T_k = \beta^k T_0$ where β was defined as $(T_f/T_o)^{1/10}$. The initial temperature was $T_o = n/\log(2)$, where $n = 4$ in this study and the final temperature was $T_f = 0.1$. The epoch-counter k was incremented after the addition of 100 members to the ensemble. Thus, as the ensemble grew, the likelihood of accepting parameter sets with a large Pareto rank decreased. To generate parameter diversity, we randomly perturbed each parameter by $\leq \pm 25\%$ at iteration of the search. In addition, we performed a local pattern search every q -iterations to minimize the residual for a single random or the worst performing objective function. The local pattern-search algorithm has been described previously [119]. From the 15,000 probable EMT models, we selected $N = 1093$ models with Pareto rank ≤ 1 for subsequent analysis. A quick estimate of the set to set correlation showed that we could expect on order 25% correlation between parameter sets in the ensemble.

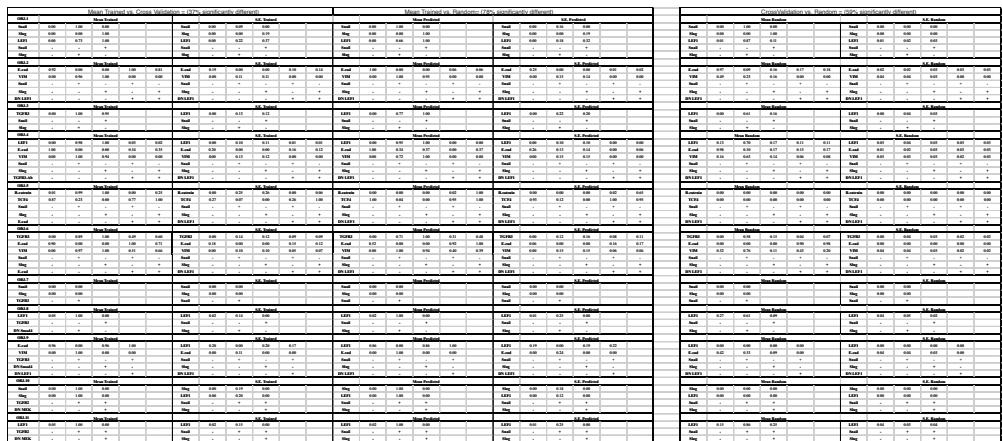


Figure S6. Training and prediction values as a function of condition for the 11 TGF- β objective functions versus a random parameter control.

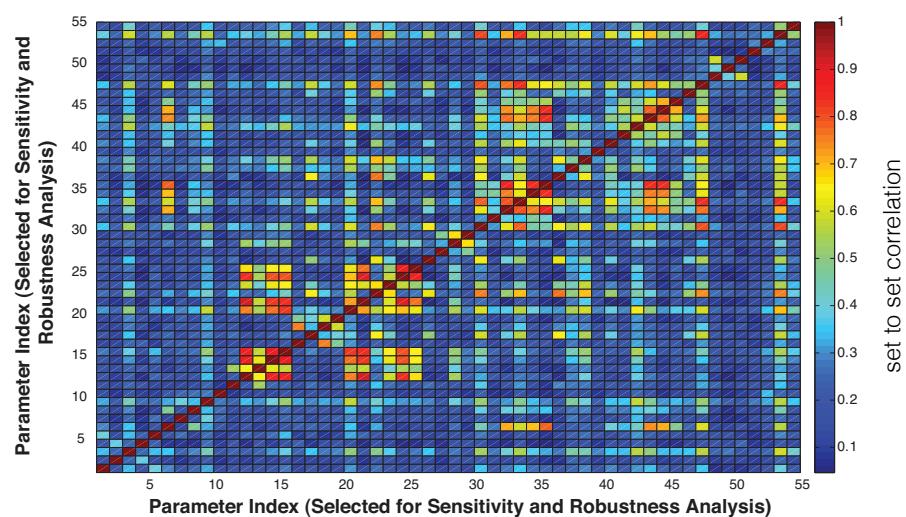


Figure S7. Parameter set to set correlation for 55 random parameter sets selected from the ensemble. Of the 55 sets selected, the average correlation between sets was less than 25% for greater than 80% of the parameter sets.

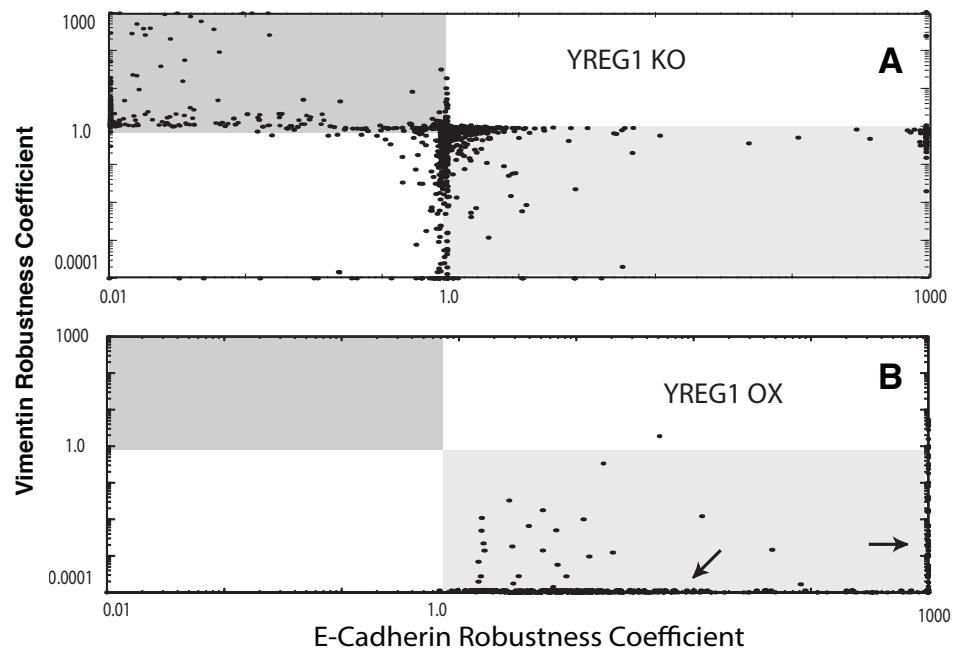


Figure S8. Robustness of E-cadherin and Vimentin expression to a knockout (A) and overexpression (B) of the hypothetical regulator 1 (YREG1) protein. Robustness coefficients were calculated for each member of the ensemble. Each point represents the response of a single model in the ensemble to either a knockout or overexpression of YREG1.

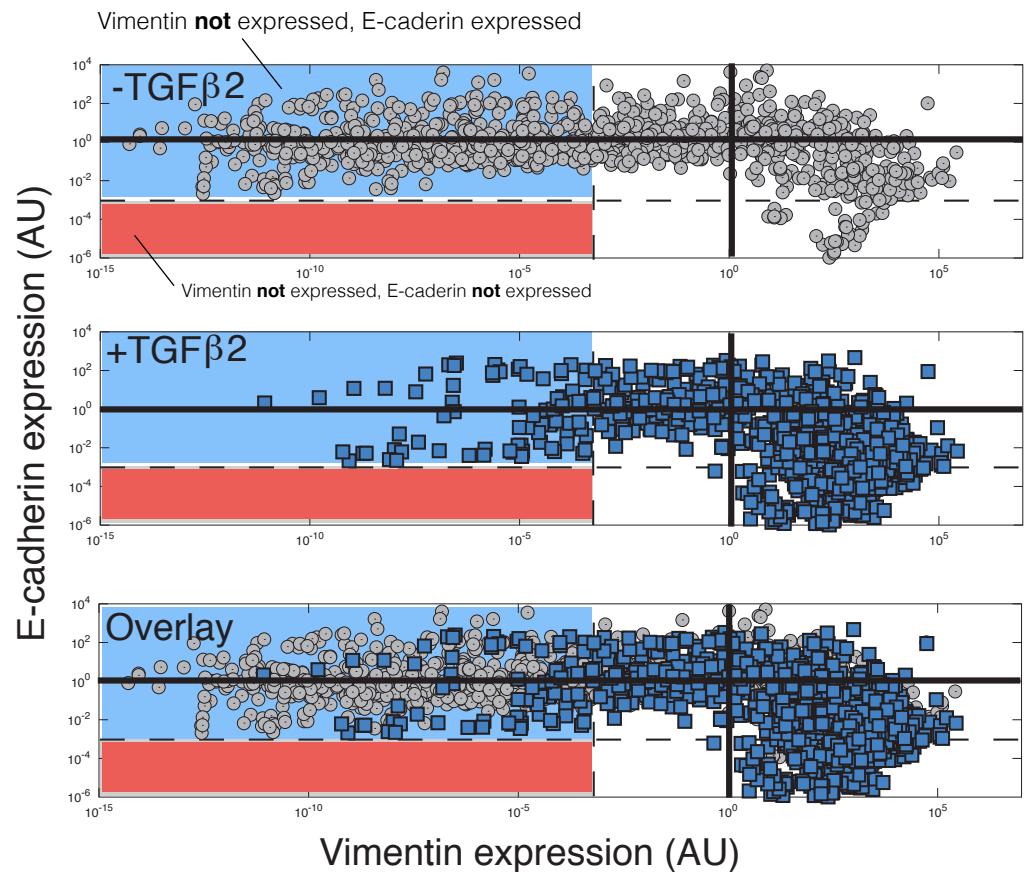


Figure S9. Steady state protein abundance for E-cadherin and Vimentin (AU) as a function of TGF- β 1/2 exposure. Top: Overlay of the model population for Vimentin (AU) and E-cadherin (AU) expression in the presence (blue) and absence (gray) of TGF- β 1/2. Middle: Vimentin (AU) and E-cadherin (AU) expression in the absence of TGF- β 1/2 showed exhibited population heterogeneity. Bottom: Vimentin (AU) and E-cadherin (AU) expression in the presence of TGF- β 1/2 moved the centroid of the population toward Vimentin (AU) and away from E-cadherin (AU) expression.