

# **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  $\beta$  (TGF- $\beta$ ) 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- $\beta$  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- $\beta$  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- $\beta$  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- $\beta$  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- $\beta$  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.

## **1 Introduction**

- 2 The epithelial to mesenchymal transition (EMT) is a broadly participating, evolutionarily  
3 conserved differentiation program essential for tissue morphogenesis, remodeling and  
4 pathological processes such as cancer Thiery (2003). During EMT polarized, tightly ad-  
5 hered epithelial cell monolayers are transformed into non-interacting motile mesenchymal  
6 cells that simultaneously degrade and synthesize extracellular matrix (ECM) components  
7 and invade into the underlying tissue space Stahl & Felsen (2001). EMT is the funda-  
8 mental initiator of developmental processes such as embryonic gastrulation and valvulo-  
9 genesis Eisenberg & Markwald (1995) (also Kalluri J Clin Invest 2009, Thiery Cell 2009).  
10 Transforming growth factor  $\beta$  (TGF- $\beta$ ) family members are important inducers of both de-

11 developmental and pathological EMT Xu *et al.* (2009), Zavadil & Böttinger (2005). Decades  
12 of research has focused on identifying molecular regulators of EMT, but almost all on a  
13 single gene and in a nearly binary yes/no level of qualitative understanding. Medici and  
14 coworkers recently identified a core signaling program by which TGF- $\beta$  isoforms induce  
15 EMT across a variety of cell lines Medici *et al.* (2006, 2008). This program involves care-  
16 fully orchestrated rounds of gene expression driven by the Smad and Snail families of  
17 transcription factors as well as other key factors such as lymphoid enhancer-binding fac-  
18 tor 1 (LEF-1), nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), and specificity  
19 protein 1 (Sp1). Coregulators such as  $\beta$ -catenin, NF- $\kappa$ B, and the ErbB family of receptor  
20 tyrosine kinases however also participate in EMT regulation, but the degree of each's in-  
21 fluence is difficult to ascertain in isolation Hardy *et al.* (2010), Huber *et al.* (2004), Jiang  
22 *et al.* (2007), Kim *et al.* (2002). EMT also exhibits complex temporal dynamics that are  
23 often intractable in gain/loss of function studies. Elucidating the master regulatory ar-  
24 chitecture controlling EMT therefore requires inclusion of these complex overlapping and  
25 non-binary behaviors.

26 Systems biology and mathematical modeling are essential tools for understanding  
27 complex developmental programs like EMT Ahmed & Nawshad (2007). Previous com-  
28 putational models of TGF- $\beta$  induced differentiation focused on single biological factors or  
29 EMT in single cells. For example, Chung *et al.*, constructed a model of TGF- $\beta$  receptor  
30 activation and Smad signaling using ordinary differential equations and mass-action ki-  
31 netics. Their model suggested that a reduction of functional TGF- $\beta$  receptors in cancer  
32 cells may lead to an attenuated Smad2 signal Chung *et al.* (2009). Similarly, Vilar *et al.*  
33 suggested that specific changes in receptor trafficking patterns could lead to phenotypes  
34 that favor tumorigenesis Vilar *et al.* (2006). Although these models provided insight into  
35 the role of receptor dynamics, EMT induction involves many other components, including  
36 competing second messengers and interconnected transcriptional regulatory loops. Inte-  
37 grating these additional scales of molecular signaling while maintaining the capacity for

38 robust prediction requires a new and expanded computational and experimental strategy.  
39 Data-driven systems approaches Cirit & Haugh (2012) or logical model formulations Mor-  
40 ris *et al.* (2011) are emerging paradigms that constrain model complexity through the in-  
41 corporation of training and validation data. These are interesting techniques because the  
42 data informs model structure (which can be expanded as more data becomes available).  
43 Alternatively, Bailey proposed more than a decade ago that a qualitative understanding  
44 of a complex biological system should not require complete definition of its structural  
45 and parametric content Bailey (2001). Shortly thereafter, Sethna and coworkers showed  
46 that complex model behavior is often controlled by only a few parameter combinations, a  
47 characteristic seemingly universal to multi-parameter models referred to as “sloppiness”  
48 Machta *et al.* (2013). Thus, reasonable model predictions are often possible with only  
49 limited parameter information. Taking advantage of this property, we developed sloppy  
50 techniques for parameter identification using ensembles of deterministic models Song  
51 *et al.* (2010). Furthermore, we proposed that the sloppy behavior of biological networks  
52 may also be seen as a source of cell-to-cell Lequieu *et al.* (2011) or even patient-to-patient  
53 heterogeneity Luan *et al.* (2010). Recently, Bayesian parameter identification techniques  
54 have also been used to explore cell-to-cell heterogeneity Hasenauer *et al.* (2011), Kalita  
55 *et al.* (2011), where a population of cells could be viewed as a dynamic ensemble of  
56 context-specific biochemical networks Creixell *et al.* (2012).

57 In this study, we developed a family of mechanistic models describing the induction  
58 of EMT by TGF- $\beta$  isoforms in the presence and absence of vascular endothelial growth  
59 factor A (VEGF-A). We incorporated mass action kinetics within an ordinary differential  
60 equation (ODE) framework to describe the EMT interaction network containing 995 gene,  
61 protein or mRNA components interconnected through 1700 interactions. A family of model  
62 parameters was estimated using 41 molecular data sets generated in DLD1 colon carci-  
63 noma, MDCKII and A375 melanoma cells using the Pareto optimal ensemble technique  
64 (POETs) multiobjective optimization algorithm. POETs identified more than 15,000 likely

65 TGF- $\beta$  induced EMT models, from which we selected approximately 1100 models for fur-  
66 ther analysis. Analysis of the model population suggested that both MCF10A and DLD1  
67 cells could exhibit phenotypic heterogeneity if treated simultaneously with TGF- $\beta$ 1/2 and  
68 VEGF-A. This heterogeneity was characterized by a significant fraction of the population  
69 being in a “hybrid state” having both high E-cadherin and high Vimentin expression. We  
70 tested these predictions using qRT-PCR and flow-cytometry studies in a variety of ex-  
71 perimental conditions. Validation studies confirmed that upwards of 45% of the cellular  
72 population could be put into the hybrid state in the presence of both TGF- $\beta$ 1/2 and VEGF-  
73 A. Moreover, this response depended upon both activation of Sp1 by MAPK and NFATc1  
74 transcriptional activity consistent with the predicted molecular signaling. Lastly, the hybrid  
75 populations of both DLD1 and MCF10A cells exhibited different functional behavior than  
76 those from either TGF- $\beta$  or VEGF-A treatment. The extent of ductal branch formation  
77 significantly increased with MCF10A cells in the hybrid phenotype, compared with cells  
78 treated with VEGF-A alone. Together, these results establish a predictive mechanistic  
79 model of EMT susceptibility, and reveal a novel signaling axis, which possibly regulates  
80 carcinoma progression through an EMT versus tubulogenesis response.

81 **Results**

82 **The model population captured key features of TGF- $\beta$  induced EMT** The EMT model  
83 architecture, based upon curated molecular connectivity, described the expression of 80  
84 genes following exposure to TGF- $\beta$  isoforms and VEGF-A (Fig. 1). The EMT model  
85 contained 995 molecular species interconnected by 1700 interactions. Model equations  
86 were formulated using mass-action kinetics within an ordinary differential equation (ODE)  
87 framework. ODEs and mass action kinetics are common tools to model biochemical path-  
88 ways Chen *et al.* (2009), Schoeberl *et al.* (2002), Tasseff *et al.* (2011). However, while  
89 ODE models can simulate complex intracellular behavior, they require estimates for model  
90 parameters which are often difficult to obtain. The EMT model had 1756 unknown model  
91 parameters, 1700 kinetic constants and 56 non-zero initial conditions. As expected, these  
92 parameters were not uniquely identifiable given the training data Gadkar *et al.* (2005).  
93 Thus, instead of identifying a single best fit (but uncertain) model, we estimated a sloppy  
94 population of models (each consistent with the training data) by simultaneously minimiz-  
95 ing the difference between model simulations and 41 molecular data sets using the Pareto  
96 Optimal Ensemble Technique (POETs). The training data were generated in DLD1 colon  
97 carcinoma, MDCKII, and A375 melanoma cells following exposure to TGF- $\beta$  isoforms  
98 Medici *et al.* (2008). We organized these data sets into 11 objective functions which  
99 were simultaneously minimized by POETs. Additionally, we used 12 molecular data sets  
100 generated in HK-2 cells following VEGF-A exposure to train VEGF-A responsive model  
101 processes Lian *et al.* (2011). To guard against overfitting, we augmented the multiobjec-  
102 tive optimization with leave-one-out cross validation to independently estimate both the  
103 training and prediction error for each objective. Thus, we generated 11 different model  
104 ensembles. Lastly, we compared model predictions with independent data sets not used  
105 during training (both at the molecular and model population levels) to evaluate the pre-  
106 dictive power of the parameter ensemble. Additional details of the signaling architecture  
107 included in the model are presented in the materials and methods and the supplement.

108 POETs generated a population of probable signaling models which captured the mul-  
109 tiple phases of EMT induction (Fig. 2). POETs sampled well over  $10^6$  probable models  
110 during each stage of the cross-validation, using a combination of both local and global  
111 random sampling. This sampling generated approximately 15,000 highly probable mod-  
112 els from which we selected  $N \simeq 1100$  models for further analysis. The selected models all  
113 had the same possible molecular connectivity, but different values for model parameters  
114 and extrinsic factors such as RNA polymerase or ribosome abundance. Model selec-  
115 tion was based upon Pareto rank, the prediction and training error across all objectives  
116 and model to model correlation (supplemental materials). The model population recapit-  
117 ulated key signaling events following TGF- $\beta$  exposure. We subdivided the response to  
118 TGF- $\beta$  exposure into two phases. First, TGF- $\beta 1/2$  signaling initiated a program which  
119 downregulated E-cadherin expression in a MAPK dependent manner while simultane-  
120 ously upregulating TGF- $\beta 3$  expression. Second, TGF- $\beta 3$  secretion initiated an autocrine  
121 feedback which upregulated the expression of mesenchymal markers such as Vimentin  
122 and key upstream transcription factors such as LEF-1 in a SMAD dependent manner.  
123 Each phase involved the hierachal expression and/or post-translational modification of  
124 several key transcription factors. During the first phase, stimulation with TGF- $\beta 1/2$  (10  
125 a.u.) activated both the SMAD and MAPK pathways. MAPK activation resulted in the  
126 phosphorylation of the transcription factor activator protein 1 (AP-1), which in-turn upreg-  
127 ulated the expression of Snail, a well established transcriptional repressor (Fig. 2A). Snail  
128 expression was MAPK-dependent; the MEK inhibitor U0126 blocked AP-1 activation and  
129 Snail expression following TGF- $\beta 1/2$  exposure (Fig. 2A, Lane 3). Similar results were ob-  
130 tained for Slug expression, confirming initial activation through the MAPK pathway (data  
131 not shown). Overexpression of either Snail or Slug upregulated TGF- $\beta 3$  expression (Fig.  
132 2C) while simultaneously downregulating E-cadherin expression (Fig. 2F). During the  
133 second phase, TGF- $\beta 3$  secretion and the subsequent autocrine signaling resulted in the  
134 upregulation of mesenchymal marker expression. The TGF- $\beta 3$  induced gene expres-

135 sion program involves a complex hierarchy of transcriptional and post-translational reg-  
136 ulatory events. Absence of E-cadherin indirectly promoted TGF- $\beta$ 3 expression through  
137 the  $\beta$ -catenin/TCF4 complex following Snail or Slug expression (Fig. 2C, Lane 2 or 3).  
138 Conversely, over-expression of E-cadherin inhibited the TGF- $\beta$ 3 autocrine production by  
139 sequestering cytosolic  $\beta$ -catenin, thereby blocking EMT (Fig. 2C, Lane 4 or 5). TGF- $\beta$ 3  
140 signaled through the Smad pathway to regulate LEF-1 expression and down-  
141 stream target EMT genes (Fig. 2G). TGF- $\beta$ 3 (10 a.u.) in combination with downstream  
142 inhibitors (DN-Smad4 and DN-LEF-1) completely inhibited Vimentin expression, while el-  
143 evating E-cadherin expression (Fig. 2H,I).

144 The predictive power of the ensemble was tested using both cross validation and by  
145 comparing simulations with data sets not used for model training. In whole, 78% of our  
146 training objectives were statistically significant (at a 95% confidence interval) compared to  
147 a randomized parameter family ( $N = 100$ ) generated from the best-fit nominal set (starting  
148 point for the optimization). Conversely, we *predicted* approximately 60% of the training  
149 objectives, at a 95% confidence interval compared to randomized parameters. The model  
150 also captured the temporal gene expression responses of E-cadherin, pSmad2, and LEF-  
151 1 to within one-standard deviation (up to the 48 hr time-point) (Fig. 2J-L). This data was  
152 not used for model training. The high predictability can be attributed to the combination  
153 of the leave-one-out cross validation scheme, diverse objective functions, and robustness  
154 of the POETs algorithm. Taken together, the model captured the key signaling events  
155 revealed by Medici *et al.* Medici *et al.* (2008) that drive the phenotypic conversion. A  
156 listing of data used for training is included in the supplement (Fig. S5 and Fig. S6).

157 **Identification of a novel LEF-1 regulator** During model identification, we found that  
158 consistent TGF- $\beta$  induced EMT required an additional regulatory protein. This protein,  
159 which we called hypothetical regulator 1 (YREG1), was required to mediate between  
160 SNAIL/SLUG transcriptional activity and the upregulation of LEF-1 expression following  
161 TGF- $\beta$ 1/2 exposure. SNAIL/SLUG are well known transcriptional repressors Dhasarathy

<sup>162</sup> *et al.* (2011), Hemavathy *et al.* (2000a,b), although there are a few studies which suggest  
<sup>163</sup> that at least SNAIL can also act as a transcriptional activator Guaita *et al.* (2002). In  
<sup>164</sup> the model, we assumed the expression of SNAIL/SLUG was likely regulated by AP1/SP1  
<sup>165</sup> Jackstadt *et al.* (2013). Thus, upon receiving a TGF- $\beta$ 1/2 signal, the model predicted en-  
<sup>166</sup> hanced SNAIL/SLUG expression, consistent with experimental observations. TGF- $\beta$ 1/2  
<sup>167</sup> stimulation also induces LEF-1 expression. However, literature evidence suggested that  
<sup>168</sup> LEF-1 expression was not strongly dependent upon AP1/SP1 activity Eastman & Gross-  
<sup>169</sup> chedl (1999). Thus, either SNAIL/SLUG are acting as inducers (contrary to substantial  
<sup>170</sup> biochemical evidence) or, they are repressing the expression of an intermediate repres-  
<sup>171</sup> sor. Given the biochemical evidence supporting SNAIL/SLUG as repressors, we cre-  
<sup>172</sup> ated YREG1 a hypothetical intermediate repressor whose expression is downregulated by  
<sup>173</sup> SNAIL/SLUG. The literature data therefore suggested that YREG1 had two transcriptional  
<sup>174</sup> targets, LEF-1 and TGF- $\beta$ 3. By adding this regulator, our simulations became consistent  
<sup>175</sup> with training and literature data. Medici *et al.* suggested a similar idea where feedback  
<sup>176</sup> between  $\beta$ -catenin and LEF-1 was likely, although this feedback had yet to be identified  
<sup>177</sup> Medici *et al.* (2008). Low levels of YREG1 expression were used in all simulations to  
<sup>178</sup> regulate the formation of the  $\beta$ -catenin-LEF-1 complex. To test the potency of YREG1,  
<sup>179</sup> we conducted knockdown and over-expression simulations following the addition of TGF-  
<sup>180</sup>  $\beta$ 1/2 (Fig. S8). In the absence of YREG1, most of the population failed to consistently  
<sup>181</sup> respond to TGF- $\beta$ 1/2 exposure compared to the wild-type (Fig. S8A). Conversely, YREG1  
<sup>182</sup> overexpression revealed an exclusively epithelial phenotype following TGF- $\beta$ 1/2 stimula-  
<sup>183</sup> tion (Fig. S8B). Overexpression of YREG1 repressed LEF-1 and TGF- $\beta$ 3 expression,  
<sup>184</sup> thereby not allowing free  $\beta$ -catenin to form the  $\beta$ -catenin-LEF-1 complex which promotes  
<sup>185</sup> mesenchymal gene expression, or SMAD activity following from autocrine TGF- $\beta$ 3 signal-  
<sup>186</sup> ing. Likewise, the abundance of the pSmad2/4-LEF-1 complex was also reduced in cells  
<sup>187</sup> overexpression YREG1, which blocked the repression of E-cadherin. Taken together,  
<sup>188</sup> we found that low YREG1 expression was necessary for stabilizing EMT, while elevated

<sup>189</sup> YREG1 levels limited the extent of EMT induction.

190 **TGF- $\beta$ 1/2 and VEGF-A exposure promotes phenotype heterogeneity through NFATc**  
191 **and phosphorylated Sp1** While we captured the central tendency of many of the molec-  
192 ular features of EMT induction following TGF- $\beta$ 1/2 exposure, an often neglected but im-  
193 portant emergent feature of developmental and pathological programs is population het-  
194 erogeneity Park *et al.* (2010). We (and others) previously hypothesized that deterministic  
195 model ensembles can interrogate population behavior, at least at a course grained level  
196 Lequieu *et al.* (2011). We tested this hypothesis by analyzing the response of the pop-  
197 ulation of EMT models to extracellular cues and then comparing this response to flow  
198 cytometry studies. We used robustness coefficients to quantify the response of the in-  
199 dividual members of the ensemble to TGF- $\beta$ 1/2 stimulation. We have previously used  
200 robustness coefficients to systematically quantify response of a system to structural or  
201 operational perturbations, for example gene deletions or the addition of a growth factor or  
202 hormone Lequieu *et al.* (2011), Song *et al.* (2010), Tasseff *et al.* (2010, 2011). Robust-  
203 ness coefficients quantify shifts in molecular marker abundance resulting from molecular  
204 or environmental perturbations relative to an unperturbed control state. Robustness coef-  
205 ficients  $\gg 1$  indicate that marker abundance increased, while robustness coefficients  $\ll$   
206 1 indicates marker abundance decreased relative to an unperturbed control. A value of  
207  $\sim 1$  indicates approximately no change in marker abundance following the perturbation.  
208 We calculated robustness coefficients for each member of the ensemble ( $N \simeq 1100$ ) for  
209 two downstream phenotypic markers, Vimentin (mesenchymal) and E-cadherin (epithe-  
210 lial) following the addition of TGF- $\beta$ 1/2 alone (Fig. 3), and VEGF-A in combination with  
211 NFATc inhibitors (Fig. 4). The absence of TGF- $\beta$ 1/2 or VEGF-A stimulation was used as  
212 the baseline for the robustness calculations.

213 We identified model subpopulations that exhibited different behaviors following expo-  
214 sure to TGF- $\beta$ 1/2 (Fig. 3A, labeled P1-P4). Analysis of the molecular signatures of  
215 these subpopulations suggested the abundance, localization and state of the Sp1, AP-1  
216 and NFATc transcription factors controlled population heterogeneity. The behavior of the

217 majority of models (>70%) was similar to subpopulation one (P1) or subpopulation two  
218 (P2) in Fig 3. These models showed the classically expected behavior, a switch from  
219 an epithelial to mesenchymal phenotype following TGF- $\beta$ 1/2 exposure. Models near P1  
220 had elevated nuclear localized phosphorylated Sp1, relative to non-induced cells (and  
221 models near P2). Elevated Sp1 activity decreased E-cadherin expression through Slug-  
222 mediated inhibition, which in turn increased Vimentin expression through TGF- $\beta$ 3 au-  
223 tocrine signaling and the liberation of  $\beta$ -catenin. Near P2, Sp1 transcriptional activity was  
224 lower than P1, leading to only modestly increased Vimentin expression and E-cadherin re-  
225 pression following TGF- $\beta$ 1/2 stimulation. Near subpopulation three (P3), reduced levels  
226 of nuclear phosphorylated AP-1, Sp1, and NFAT (resulting from the loss of ERK kinase ac-  
227 tivity) were responsible for Vimentin *repression* relative to the control. However, the most  
228 biologically interesting behavior was exhibited by subpopulation four (P4). Models near  
229 P4 had elevated Sp1 and NFAT transcriptional activity, which increased *both* Vimentin and  
230 E-cadherin expression. Analysis of these hypothetical cells suggested they had *abnormal*  
231 signaling; deregulated NFAT expression and nuclear localization promoted E-cadherin ex-  
232 pression while TGF- $\beta$ 1/2 induced Sp1 action promoted Vimentin expression. Analysis  
233 of the connectivity and information flow through the signaling architecture suggested that  
234 Sp1 and NFAT action could be manipulated *independently* by simultaneous TGF- $\beta$ 1/2  
235 and VEGF-A stimulation (Fig. S1).

236 To test this hypothesis, we simulated the response of the network to TGF- $\beta$ 1/2 and  
237 VEGF-A treatment with and without NFATc inhibitors (Fig. 4). As expected, stimulation  
238 with VEGF-A (50 a.u.) maintained an epithelial population (Q4-43.6%), while TGF- $\beta$ 1/2  
239 (10 a.u.) exposure shifted the population from an epithelial (Q4-5.5%) to a mesenchymal  
240 (Q1-45.6%) phenotype (Fig. 4A and Fig. 4B). On the other hand, combined stimula-  
241 tion with TGF- $\beta$ 1/2 (10 a.u.) and VEGF-A (50 a.u.) increased both E-cadherin and  
242 Vimentin expression (Q2-45.3%), resulting in a hybrid phenotype with both epithelial and  
243 mesenchymal characteristics (Fig. 4C). To better understand this hybrid response, we

244 quantified the simulated protein levels for E-cadherin, Vimentin, phosphorylated nuclear  
245 Sp1, nuclear NFATc1,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and Slug as a function of condition  
246 (Fig. S2A-C). Vimentin expression was correlated with high levels of nuclear phospho-  
247 lyated Sp1, following TGF- $\beta$ 1/2 exposure. Conversely, elevated E-cadherin expression  
248 depended upon the activity of NFAT transcription factors downstream of VEGF-A stimula-  
249 tion. To further isolate the role of NFAT on this hybrid state, we simulated the inhibition of  
250 NFAT transcriptional activity across all conditions (all else being equal). NFAT inhibition in  
251 combination with VEGF-A treatment blocked all E-cadherin positive sets (Fig. 4D). Like-  
252 wise, TGF- $\beta$ 1/2 treatment in combination with NFATc inhibition also resulted in the loss  
253 of E-cadherin expression (Fig. 4E). Lastly, NFATc inhibition in combination with simu-  
254 taneous TGF- $\beta$ 1/2 and VEGF-A exposure repressed nearly all E-cadherin expression,  
255 shifting nearly the entire population towards a mesenchymal phenotype (Fig. 4F). Taken  
256 together, high levels of nuclear localized phosphorylated Sp1 correlated with Vimentin ex-  
257 pression, while NFATc transcriptional activity was predicted to be critical for maintaining  
258 E-cadherin expression.

259 **Combined TGF- $\beta$ 2 and VEGF-A exposure drives heterogeneity in MCF10A and**  
260 **DLD1 cells** The EMT model simulations suggested the transcriptional activity of NFATc  
261 and Sp1 could be independently tuned to generate a hybrid cell population with both  
262 epithelial and mesenchymal characteristics. To test this hypothesis, we exposed either  
263 quiescent epithelial (MCF10, (Fig. 5)) or transformed epithelial cells (DLD1, (Fig. S3))  
264 to combinations of TGF- $\beta$ 1/2 and/or VEGF-A. As expected, treatment with TGF- $\beta$ 1/2  
265 (10ng/ml) increased Slug and Vimentin expression, while repressing E-cadherin expres-  
266 sion both at the transcript and protein levels in MCF10A (Fig. 5A-B) and DLD1 cells (Fig.  
267 S4C, Fig S3 D,E). Both MCF10A (Fig. 5C) and DLD1 cells (Fig. S3E,G) transitioned  
268 from quiescent cobblestone morphology to spread spindle shapes, consistent with EMT.  
269 As predicted, we found increased nuclear localization of phosphorylated Sp1 following  
270 TGF- $\beta$ 1/2 stimulation in both MCF10A (Fig. 5B,C) and DLD1 cells (Fig. S3E,F). Con-

sistent 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- $\beta$ 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- $\beta$ 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- $\beta$ 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- $\beta$ 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- $\beta$ 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- $\beta$ 1/2 in the presence or absence of VIVIT, a soluble peptide inhibitor of NFATc transcriptional activity Aramburu *et al.* (1999). Treatment with VEGF-A (50ng/ml) and VIVIT (10 $\mu$ M) in MCF10A cells significantly reduced E-cadherin expression compared to VEGF-A alone (Fig 5D,E). Co-treatment with VIVIT and TGF- $\beta$ 1/2 did not enhance EMT capacity of MCF10A cells above that of TGF- $\beta$ 1/2 alone (Fig 5A,B,E). Likewise, VIVIT in combination with both TGF- $\beta$ 1/2 and

298 VEGF-A resulted in a loss of E-cadherin gene and protein expression, while Slug and  
299 Vimentin levels remained increased (Fig. 5D,E ). Quantitative flow cytometry confirmed  
300 these results in both MCF10A (Fig. 5F) and DLD1 cells (Fig. S4C). Both epithelial cell  
301 lines initially had high levels of E-cadherin expression, and low vimentin abundance (Q1-  
302 99.5%), but both MCF10A and DLD1 cells shifted from an epithelial to mesenchymal  
303 phenotype (Q1-33.4%, Q4-42.8%) following TGF- $\beta$ 1/2 exposure. As expected, NFATc  
304 nuclear localization was repressed with VIVIT treatment regardless of ligand stimulation,  
305 while the abundance of nuclear phosphorylated Sp1 increased for both TGF- $\beta$ 1/2 and  
306 TGF- $\beta$ 1/2 + VIVIT conditions (Fig. 5D,E). Combined TGF- $\beta$ 1/2 and VEGF-A increased  
307 both Vimentin and E-cadherin expression (Q1-42.1%, Q2-52.3%) compared to TGF- $\beta$ 1/2  
308 alone. Together, these results demonstrate that NFATc and phosphorylated Sp1 are criti-  
309 cal for regulating E-cadherin and Vimentin expression during phenotype heterogeneity in  
310 MCF10A and DLD1.

311 **Ductal branching during acini formation is dependent upon phenotype heterogene-  
312 ity in MCF10A and DLD1 cells** We finally employed established three-dimensional  
313 (3D) *in vitro* models of invasion, migration, compaction, and tubulogenesis Dhimolea  
314 *et al.* (2010) to determine the functional consequences of the hybrid phenotype (Fig. 6).  
315 MCF10A and DLD1 cells were aggregated via hanging drop, placed on the surface of a  
316 collagen gel, and cultured for 72 hrs under various biochemical treatments. TGF- $\beta$ 1/2  
317 stimulation significantly enhanced cell matrix invasion and matrix compaction, while in  
318 contrast VEGF-A stimulation promoted surface migration but no invasion or compaction  
319 (Fig. 6B-D). Interestingly, combined TGF- $\beta$ 1/2 and VEGF-A stimulation significantly in-  
320 creased cell migration potential above that of VEGF-A alone while maintaining 3D matrix  
321 compaction, though with decreased magnitude compared to TGF- $\beta$ 1/2 alone. Inhibi-  
322 tion of NFATc transcriptional activity by VIVIT decreased migration following treatment  
323 with VEGF-A alone (Fig. 6B). Co-treatment of VIVIT significantly decreased migration,  
324 while complementarily increasing invasion and compaction, when MCF10A cells were

325 stimulated with both VEGF-A and TGF- $\beta$ 1/2 (Fig. 6B-D). The responses of DLD1 cells  
326 followed a similar trend to MCF10A, although the magnitudes of migration, invasion, and  
327 compaction were less. Cell circularity within 3D gels strongly and negatively correlated  
328 with both invasion and compaction regardless of treatment (Fig. 6E). CircularitY refers  
329 to the morphology of the cells. In general, a quiescent epithelial cells assumes a circular  
330 morphology in culture, while an active mesenchymal cell is highly elongated. The circular-  
331 ity index, a common means of quantifying cell morphology, relates cell area to perimeter.  
332 A perfect circle has a circularity index equal to 1.0, while a straight line has a circularity  
333 index equal to 0.0, see Butcher et al. Butcher *et al.* (2004). TGF- $\beta$ 1/2 treatment alone  
334 resulted in irregular and spindle shaped morphology, while VEGF-A exposure promoted  
335 round quiescent cells (Fig. 6A). Combined VEGF-A and TGF- $\beta$ 1/2 promoted morphology  
336 between these extremes. VIVIT mediated NFATc inhibition significantly reduced the cir-  
337 cularity index, similar to TGF- $\beta$ 1/2 treatment (Fig. 6F). VEGF-A treatment also induced  
338 the formation of tubular structures (acini), but the number of tubular branches relative to  
339 total acini was significantly increased upon combined TGF- $\beta$ 1/2 and VEGF-A. No tubular  
340 structures were identified within the DLD1 constructs during the 7 day tubulogenesis end-  
341 points, supporting that MCF10A and DLD1 cells have some cell-type specific EMT sensi-  
342 tivity despite their underlying competency for acquiring a heterogeneous phenotype. This  
343 suggests that initial EMT sensitivity of a cell influences downstream functional response  
344 from TGF- $\beta$  and VEGFA stimulation. Together, these results establish that VEGF-A and  
345 TGF- $\beta$ 1/2 ligand concentrations potentiate between acini and ductal branch formation in  
346 3D culture, and are dependent upon NFATc activity.

347 **Discussion**

348 In this study, we developed a family of mechanistic models describing the induction of  
349 EMT by TGF- $\beta$  isoforms in the presence and absence of VEGF-A. The signaling archi-  
350 tecture encoded in the model, which contained 995 molecular species interconnected  
351 by 1700 interactions, described the expression of 80 genes in response to growth fac-  
352 tor stimulation. This simulation incorporates an unprecedented level of detail compared  
353 to previous models, but as a consequence created a large number of unknown model  
354 parameters. Because these parameters could not be estimated uniquely apriori, we es-  
355 timated an ensemble of likely parameters using the POETs multiobjective optimization  
356 framework. The model population was trained and cross-validated to prescribe biolog-  
357 ical significance using 41 data sets generated in DLD1 colon carcinoma, MDCKII, and  
358 A375 melanoma cell lines Medici *et al.* (2008). POETs generated  $> 15,000$  probable pa-  
359 rameter sets using this data, from which we selected  $N \simeq 1100$  for subsequent analysis.  
360 Analysis of this population predicted possible phenotypic modes (and their associated  
361 signaling) that cells could exhibit when stimulated with TGF- $\beta$  and/or VEGF-A. The most  
362 novel hypothesis generated from the analysis was that cells could operate in a hybrid  
363 state defined by both epithelial and mesenchymal traits when stimulated simultaneously  
364 with TGF- $\beta$  and VEGF-A. We tested this hypothesis in MCF10A and DLD1 cells stimu-  
365 lated with combinations of TGF- $\beta$  and VEGF-A. As expected, in the presence of TGF- $\beta$   
366 or VEGF-A alone, MCF10A and DLD1 cells were either mesenchymal or epithelial, re-  
367 spectively. However, with both TGF- $\beta$  and VEGF-A, MCF10A and DLD1 cells exhibited a  
368 hybrid phenotype, having both epithelial and mesenchymal characteristics. Furthermore,  
369 we found that functional traits such as tubulogenesis and ductal branching were different  
370 for cells in this hybrid phenotype. Together, this study established a predictive model of  
371 EMT induction, determined that deterministic model ensembles could predict population  
372 heterogeneity, and proved the existence of a unique hybrid phenotype resulting from the  
373 simultaneous integration of extracellular growth factor signals.

374 Cells routinely process a multitude of signals simultaneously, especially when coordi-  
375 nating developmental or pathological programs. For example, oncogenic cells integrate  
376 both mechanical and chemical cues in their local microenvironment during tumorigenesis,  
377 including cytokines VEGF and TGF- $\beta$  Hong *et al.* (2013). VEGF-A mediates patholog-  
378 ical angiogenic remodeling of tumors Nagy *et al.* (2007), while TGF- $\beta$  can elicit both  
379 protective and oncogenic responses Ferrara (2002), Willis & Borok (2007). While much  
380 research has tested signaling pathways individually, far less is understood about combi-  
381 natorial stimulation, such as with both VEGF-A and TGF- $\beta$ . Recent *in vitro* and *in vivo*  
382 evidence has suggested that epithelial cells can exhibit heterogeneous phenotypes in ad-  
383 dition to classically defined epithelial or mesenchymal states Polyak & Weinberg (2009),  
384 Strauss *et al.* (2011). For example, expression profiling in human epithelial cancer cell  
385 lines demonstrated a spectrum of phenotypes, including some that expressed both E-  
386 cadherin and Vimentin simultaneously Neve *et al.* (2006), Welch-Reardon *et al.* (2014).  
387 Zajchowski *et al.*, speculated that these expression profiles were somehow important for  
388 maintaining epithelial properties, while simultaneously allowing other functional behavior  
389 such as proliferation and migration Zajchowski *et al.* (2001). Whether and how heteroge-  
390 neous phenotypes arise and participate in cancer progression, as well as their response  
391 to pharmacological inhibition are fundamental questions that should receive increased at-  
392 tention. In this study, we determined that a hybrid phenotype could be obtained through  
393 combined treatment with VEGF-A and TGF- $\beta$ , both common factors localized in the tu-  
394 mor microenvironment. Furthermore, our systematic simulation-experimentation strategy  
395 identified that the transcriptional activity of Sp1 and NFATc were the critical factors con-  
396 trolling this phenotypic heterogeneity. Several studies have highlighted the importance  
397 of NFATc as a key transcription factor involved in cell growth, survival, invasion, angio-  
398 genesis and cancer Mancini & Toker (2009). For example, proliferation and anchorage-  
399 independent growth of pancreatic tumor cells is dependent on calcineurin and NFATc1  
400 activity, consistent with the high levels of nuclear NFATc1 found in pancreatic tumors

401 Singh *et al.* (2010). Likewise, our results found that VEGF-A was a potent inducer of  
402 NFATc1 expression, which may be required for epithelial cell migration and tubulogenesis.  
403 Although specific NFATc isoforms were not distinguished in the model, our simulations  
404 suggested that NFATc transcriptional activity was capable of maintaining epithelial traits,  
405 even during TGF- $\beta$  induced EMT. Experimentally, we found that E-cadherin expression  
406 was dependent upon NFATc dephosphorylation in response to simultaneous VEGF-A and  
407 TGF- $\beta$ 1/2 treatment. Thus, these results support the hypothesis that NFATc activity plays  
408 a critical role in maintaining cell-cell contacts, even during partial EMT.

409       Epithelial cells reproduce tissue-like organization when grown in a three-dimensional  
410 extracellular matrix (ECM) environment, and therefore are an attractive model to study  
411 morphogenic mechanisms. It is well established that MCF10A cells form structures that  
412 closely resemble acini (multi-lobed cluster of cells) in three-dimensional *in vitro* cultures  
413 Debnath *et al.* (2003). It has been postulated that a cellular response reminiscent of partial  
414 EMT underlies this process, stimulating further branching and formation of acini Pearson  
415 & Hunter (2007). Normally well controlled process such as tubulogenesis can be co-opted  
416 by cancer cells to break away from a primary lesion and invade through the surrounding  
417 stroma O'Brien *et al.* (2004). However, by retaining a transient hybrid EMT-like state, clus-  
418 ters of these tube-forming tumor cells can reform at a high rate after invasion, possibly  
419 explaining why invasive human carcinomas frequently appear to be cellular collections  
420 with varying degrees of gland-like differentiation Debnath & Brugge (2005). In this study,  
421 we showed that our predicted hybrid phenotype generated by simultaneous treatment  
422 of epithelial cells with VEGF-A and TGF- $\beta$  possessed altered migration and invasion,  
423 which enhanced tubular branching. A salient feature of this behavior, however, was the  
424 retention of cell-cell contacts that allowed cells to migrate without completely dissociat-  
425 ing from their neighbors. Thus, our results support a mechanism in which hybrid cells  
426 can maintain some functional characteristics of epithelial cells such as cell-cell adhesion,  
427 which are normally lost in a fully differentiated mesenchymal state. The tumor microen-

428 vironment contains many soluble signals simultaneously, including VEGF and TGF- $\beta$ .  
429 Thus, it is likely that some cancerous epithelial cells could exhibit hybrid EMT pheno-  
430 typic states. This may explain why fibroblastoid morphology, a classical feature of EMT,  
431 is not commonly observed in human carcinomas Debnath & Brugge (2005). This study  
432 focused on the combinatorial effects of two very different ligand families present together  
433 in the tumor environment. Additional modeling studies are required to unravel the global  
434 response of epithelial cells to the full spectrum of chemical, substrate, and mechanical  
435 cues. The simulation strategy presented here is readily adaptable to larger species sets,  
436 with the major advantage that experimentally testable hypotheses can be generated re-  
437 garding how signals get integrated to produce global cellular response. Furthermore, by  
438 simulating multiple ensembles of parameter sets, subpopulations across a constellation of  
439 phenotypes can be created and mined for common and/or divergent signaling character-  
440 istics. This is a significant advantage over forced convergence to a single unique solution  
441 and thereby generating a potentially non-physiological homogeneous population.

442 The deterministic population of EMT models predicted heterogeneous behavior that  
443 was qualitatively consistent with experimental studies. There is a diversity of algorithmic  
444 approaches to estimate model parameters Moles *et al.* (2003), as well as many strate-  
445 gies to integrate model identification with experimental design Rodriguez-Fernandez *et al.*  
446 (2013), Villaverde & Banga (2014). However, despite these advances, the identification  
447 of models describing intracellular network behavior remains challenging. There are dif-  
448 ferent schools of thought to deal with this challenge. One school has focused on model  
449 reduction. Data-driven approaches Cirit & Haugh (2012), boolean Choi *et al.* (2012) or  
450 other logical model formulations Morris *et al.* (2011), Terfve *et al.* (2012) are emerging  
451 paradigms that constrain model complexity by the availability of the training and validation  
452 data. Other techniques such as constraints based modeling, which is commonly used  
453 to model metabolic networks, have also been applied to model transcriptional networks,  
454 although primarily in lower eukaryotes and prokaryotes Hyduke & Palsson (2010). These

455 techniques (and many others, see review Wayman & Varner (2013)) are certainly exciting,  
456 with many interesting properties. However, we used the traditional approach of mass ac-  
457 tion kinetics within an ordinary differential equation framework. The identification problem  
458 for the EMT model was massively underdetermined. This is not uncommon for differen-  
459 tial equation models, especially those that are highly mechanistic. Of course, we could  
460 have discarded mechanism or reduced the model scope to decrease the complexity of  
461 the identification problem. However, a central criticism leveled by biologists is that model  
462 simplification is often done at the cost of biological reality, or done for reasons of com-  
463 putational expediency Sainani (2012). To avoid this criticism, we systematically identified  
464 an ensemble of likely models each consistent with the training data, instead of a single  
465 but uncertain best fit model. Previously, we (and others) have suggested that determin-  
466 istic ensembles could model heterogeneous populations in situations where stochastic  
467 computation was not feasible Lequieu *et al.* (2011). Population heterogeneity using deter-  
468 ministic model families has previously been explored for bacterial growth in batch cultures  
469 Lee *et al.* (2009). In that case, distributions were generated because the model parame-  
470 ters varied over the ensemble, i.e., extrinsic noise led to population heterogeneity. In this  
471 study, parameters controlling physical interactions such as disassociation rates or the rate  
472 of assembly or degradation of macromolecular machinery such as ribosomes were widely  
473 distributed over the ensemble. Population heterogeneity can also arise from intrinsic ther-  
474 mal fluctuations, which are not captured by a deterministic population of models Swain  
475 *et al.* (2002). Thus, deterministic ensembles, provide a coarse-grained or extrinsic-only  
476 ability to simulate population diversity. Despite this limitation, our prediction of phenotypic  
477 heterogeneity (and the underlying signaling events responsible for the heterogeneity) was  
478 consistent with experimental observations. This suggested that deterministic ensembles  
479 could simulate disease or developmental processes in which heterogeneity plays an im-  
480 portant role, without having to resort to stochastic simulation.

481 A common criticism of ODE modeling has been the poorly characterized effect of

482 structural and parametric uncertainty. In this study, parametric uncertainty was addressed  
483 by developing an ensemble of probable models instead of a single best-fit but uncertain  
484 model using multiobjective optimization. While computationally complex, multiobjective  
485 optimization is an important tool to address qualitative conflicts in training data that arise  
486 from experimental error or cell line artifacts Handl *et al.* (2007). On the other hand, struc-  
487 tural uncertainty is defined as uncertainty in the biological connectivity. The EMT model  
488 connectivity was assembled from an extensive literature review. However, several poten-  
489 tially important signaling mechanisms were not included. First, we identified a potential  
490 gap in biological knowledge surrounding the regulation of LEF-1 expression, that was  
491 filled by the addition of the hypothetical YREG1 transcriptional repressor. The LEF-1 tran-  
492 scription factor is expressed in tissues that undergo EMT during embryogenesis Nawshad  
493 & Hay (2003), Vega *et al.* (2004), and has been suggested to promote an invasive phe-  
494 notype in cancer cells Cano *et al.* (2000), Kim *et al.* (2002). Low levels of YREG1 were  
495 important for stabilizing the interaction between LEF-1 and  $\beta$ -catenin, while elevated lev-  
496 els inhibited EMT by downregulating LEF-1 transcriptional activity. Recent evidence has  
497 established a complex role of Amino terminal Enhancer of Split (AES) and Groucho/TLE  
498 on suppressing LEF-1 activity. AES opposes LEF-1 transcriptional activation while Grou-  
499 cho/TLE binds with LEF-1 for a histone deacetylase repression. In addition,  $\beta$ -catenin  
500 directly displaces Groucho/TLE repressors from TCF/LEF-1 in Wnt-mediated transcrip-  
501 tion activation Arce *et al.* (2009), Grumolato *et al.* (2013). Our model agrees with this  
502 newly discovered feedback system, as YREG1 regulates LEF-1 activity leading to EMT  
503 stabilization. Secondly, we should revisit the role of GSK-3 $\beta$ . GSK-3 $\beta$  is an important  
504 regulator which controls the abundance of both Snail and  $\beta$ -catenin through the ubiquitin-  
505 proteasome pathway Larue & Bellacosa (2005), Zhou *et al.* (2004). Specifically to our  
506 model, expression of Snail increases through 72 hrs. In contrast, experimental data has  
507 shown that activity of Snail peaks at 24 hrs which may be controlled by the GSK-3 $\beta$  com-  
508 plex Medici *et al.* (2006). Recent evidence has also suggested an essential role of NF- $\kappa$ B

509 in epithelial transformation. NF- $\kappa$ B may influence Snail expression through the AKT path-  
510 way and directly stabilize Snail activity Wu *et al.* (2009). This is particularly important  
511 for integrating inflammation pathways, such as interleukin-6 (IL-6) and tumor necrosis  
512 factor- $\alpha$  (TNF- $\alpha$ ), which have been linked to EMT in pathological conditions Sullivan *et al.*  
513 (2009). Other pathways such as Notch have also been shown to act synergistically with  
514 TGF- $\beta$  to express Slug in the developing embryo Niessen *et al.* (2008). Lastly, while we  
515 have modeled classical protein signaling, we have not considered the role of regulatory  
516 RNAs on EMT. There is growing evidence that microRNAs (miRNAs) play a strong role  
517 in EMT, where several miRNAs, for example miR-21 and miR-31 are strongly associated  
518 with TGF- $\beta$  exposure Bullock *et al.* (2012). Addressing missing structural components  
519 like these, could generate more insight into TGF- $\beta$  signaling and its role in phenotypic  
520 transformation.

521 **Materials and Methods**

522 The simulation code and parameter ensemble used in this study can be downloaded from  
523 GitHub ([https://github.com/jeffreyvarner/TGFb-VEGFA-Model\\_v1.git](https://github.com/jeffreyvarner/TGFb-VEGFA-Model_v1.git)).

524 **Signaling network connectivity** The EMT model described the gene expression pro-  
525 gram resulting from TGF- $\beta$  and VEGF-A signaling in a prototypical epithelial cell. The  
526 TGF- $\beta$ -EMT network contained 995 nodes (proteins, mRNA or genes) interconnected by  
527 1700 interactions. The network connectivity was curated from more than 40 primary liter-  
528 ature sources in combination with on-line databases Jensen *et al.* (2009), Linding *et al.*  
529 (2007). The model interactome was not specific to a single epithelial cell line. Rather, we  
530 assembled canonical pathways involved in TGF- $\beta$  and VEGF-A signaling, defaulting to  
531 human connectivity when possible. Using a canonical architecture allowed us to explore  
532 general features of TGF- $\beta$  induced EMT without cell line specific artifacts. On the other  
533 hand, because of the canonical architecture, we evaluated the simulation conclusions in  
534 several cell lines to test the generality of our conclusions.

535 Our signaling network reconstruction was based on Medici *et al.* who identified the  
536 pathways through which MDCKII, DLD1 colon carcinoma, and A375 melanoma cells tran-  
537 sition towards a mesenchymal phenotype Medici *et al.* (2008). Sequential activation of  
538 MAPK and Smad pathways were initiated upon addition of TGF $\beta$ 1/2. Briefly, TGF $\beta$ 2 sig-  
539 nals through the RAS-RAF-MEK-ERK pathway to up-regulate Snail and Slug expression  
540 Medici *et al.* (2006). Snail, a known repressor of junctional proteins, inhibits the expres-  
541 sion of E-cadherin Cano *et al.* (2000). This initial repression of E-cadherin leads to a  
542 release of  $\beta$ -catenin from the cell membrane. Cytosolic  $\beta$ -catenin can then translocate to  
543 the nucleus and form transcriptional complexes with TCF-4 to drive TGF $\beta$ 3 expression  
544 Medici *et al.* (2008). TGF $\beta$ 3 signals to the cells interior by binding to type II receptors,  
545 which form heterodimers with type I receptors (ALK5) Derynck & Zhang (2003). This  
546 activates the receptors serine/threonine kinase activity to phosphorylate and activate the  
547 receptor Smads 2/3 Massagué *et al.* (2005). Phosphorylated Smads 2/3 (pSmad2/3)

548 form heterodimers with partner Smad4 and translocate to the nucleus. pSmads com-  
549 plexes up-regulate other transcription factors, such as LEF-1. The pSmad2/4/LEF-1 has  
550 been shown to directly repress the E-cadherin gene Nawshad *et al.* (2007). LEF-1 also  
551 binds with  $\beta$ -catenin to upregulate mesenchymal proteins such as fibronectin Medici *et al.*  
552 (2011). The EMT gene expression program was initiated by the binding of TGF- $\beta$  iso-  
553 forms to TGF- $\beta$  surface receptors. Binding of extracellular TGF- $\beta$ 1/2 with TGF- $\beta$  sur-  
554 face receptors I/II (TGF- $\beta$ R-I/II) initiates the assembly of adapter complexes which starts  
555 the downstream signaling program. In the model, TGF- $\beta$ 1/2 binds TGF- $\beta$ R-I/II followed  
556 by the recruitment of activin receptor-like kinase 1 (ALK1) and TGF- $\beta$  surface receptor  
557 III (TGF- $\beta$ R-III) to form the activated receptor complex Derynck & Zhang (2003). Alter-  
558 natively, we also included activin receptor-like kinase 5 (ALK5) recruitment in combination  
559 with Endoglin and TGF- $\beta$ R-III as a second (redundant) activated receptor complex Gatza  
560 *et al.* (2010). Complex assembly activates the serine/threonine kinase activity on the re-  
561 ceptor, leading to the recruitment and phosphorylation of Smad partners Massagué *et al.*  
562 (2005). Phosphorylated Smads2/3 (pSmad2/3) form heterodimers with partner Smad4  
563 and then translocate to the nucleus where they act as both transcriptional activators and  
564 repressors. Nuclear pSmad2/3-Smad4 form transcriptional complexes with several genes  
565 in the model including lymphoid enhancer-binding factor 1 (*LEF-1*), Nuclear factor of ac-  
566 tivated T-cells, cytoplasmic 1 (*NFACT1*), and Specificity Protein 1 (*SP1*). On the other  
567 hand, nuclear pSmad2/3-Smad4 represses (in combination with the LEF-1 protein) the  
568 expression of E-cadherin (*Cdh1*) Nawshad *et al.* (2007) and Cadherin 5, type 2 (VE-  
569 Cadherin encoded by *Cdh5*). Repression of E-cadherin expression is the central event in  
570 the transition from an epithelial to a mesenchymal phenotype Cano *et al.* (2000). How-  
571 ever, this transition is not solely driven by transcriptional events. At the protein level, the  
572 repression of E-cadherin leads to a release of  $\beta$ -catenin from cell membrane. Cytosolic  $\beta$ -  
573 catenin then translocates to the nucleus and forms transcriptionally-active complexes with  
574 immunoglobulin transcription factor 2 (TCF-4) to drive TGF- $\beta$ 3 expression Medici *et al.*

575 (2008). Simultaneously, ERK1/2-mediated phosphorylation of the AP1 and Sp1 transcription  
 576 factors can also regulate transcriptional complexes involving NFAT, Slug, and Smads.  
 577 Lastly, canonical pathways for processing extracellular VEGF-A, BMP and Wnt signals,  
 578 in addition to the PI3K pathway were also included in the model. Additional information  
 579 about the interactions included in the model, along with the Systems Biology Markup Lan-  
 580 guage (SBML) file encoding these interactions are included in the supplemental materials.

581 **Formulation, solution and analysis of the EMT model equations** EMT was modeled  
 582 using mass-action kinetics within an ordinary differential equation (ODE) framework:

$$\frac{dx}{dt} = S \cdot r(x, k) \quad x(t_0) = x_0 \quad (1)$$

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

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

594 The set  $\{R_q\}$  denotes reactants for reaction  $q$ , while  $\sigma_{jq}$  denotes the stoichiometric coeffi-  
 595 cient (element of the matrix  $S$ ) governing species  $j$  in reaction  $q$ . The quantity  $k_q$  denotes  
 596 the rate constant (unknown) governing reaction  $q$ . Model equations were generated in the

597 C-programming language using the UNIVERSAL code generator, starting from an text-  
598 based input file (supplemental materials). UNIVERSAL, an open source Objective-C/Java  
599 code generator, is available as a Google Code project (<http://code.google.com/p/universal->  
600 code-generator/). Model equations were solved using the CVODE solver in the SUNDI-  
601 ALS library Hindmarsh *et al.* (2005) on an Apple workstation (Apple, Cupertino, CA) as  
602 previously described Tasseff *et al.* (2011).

603 *Estimation of model parameters using multiobjective optimization.* The EMT model had  
604 1756 unknown parameters (1700 kinetic constants and 56 non-zero initial conditions)  
605 which were not uniquely identifiable given the training data. Instead, we estimated a pop-  
606 ulation of likely models (each consistent with the training data) using 41 data sets gen-  
607 erated in DLD1 colon carcinoma, MDCKII, and A375 melanoma cells taken from Medici  
608 *et al.* Medici *et al.* (2008). We used the Pareto Optimal Ensemble Technique (POETs)  
609 multiobjective optimization framework in combination with leave-one-out cross-validation  
610 to estimate an ensemble of model parameters Song *et al.* (2010). Cross-validation was  
611 used to calculate both training and prediction error during the parameter estimation pro-  
612 cedure Kohavi (1995). The 41 intracellular protein and mRNA data-sets used for iden-  
613 tification were organized into 11 objective functions. These 11 objective functions were  
614 then partitioned, where each partition contained ten training objectives and one validation  
615 objective. The training and validation data were Western blots. Thus, all model simula-  
616 tions were in arbitrary units. However, POETs does allow a soft constraint on the order  
617 of magnitude of the model concentration scale. In this study, we assumed the natural  
618 model concentration scale was pmol/L. We did not place a lower bound on model states.  
619 However, based on the pmol/L natural scale, we treated all values less than  $10^{-3}$  as zero  
620 (or no expression).

621 *Robustness coefficients.* Robustness coefficients were calculated as shown previously  
622 Lequieu *et al.* (2011), Tasseff *et al.* (2011). Robustness coefficients denoted by  $\alpha(i, j, t_o, t_f)$ :

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

623 quantify the response of a marker to a structural or operational perturbation to the net-  
624 work architecture. Here  $t_o$  and  $t_f$  denote the initial and final simulation time respectively,  
625 while  $i$  and  $j$  denote the indices for the marker and the perturbation respectively. A value  
626 of  $\alpha(i, j, t_o, t_f) > 1$ , indicates increased marker abundance, while  $\alpha(i, j, t_o, t_f) < 1$  indi-  
627 cates decreased marker abundance following perturbation  $j$ . If  $\alpha(i, j, t_o, t_f) \sim 1$  the  $j$ th  
628 perturbation does not influence the abundance of marker  $i$ . Robustness coefficients were  
629 calculated for each member of the ensemble ( $N \simeq 1100$ ).

630 **Cell culture and experimental interrogation** DLD1 colon carcinoma, MCF10A, and  
631 HUVEC were acquired from the American Tissue Culture Collection (Manassas, VA).  
632 Cells were grown in culture with RPMI 1640 medium with 10% fetal bovine serum and  
633 1% penicillin/streptomycin for DLD1, EBM-2 supplemented with EGM-2, 5% fetal bovine  
634 serum, and 1% penicillin/streptomycin for HUVEC, or MGEM 2 supplemented with insulin,  
635 bovine pituitary extract, cholera toxin, hEGF, hydrocortisone, 5% horse serum, and 1%  
636 penicillin/streptomycin for MCF10A. Cells were serum starved for 24 hours and removed  
637 from all experimental conditions. Recombinant VEGFA165 was also removed from cul-  
638 ture medium prior to experimentation. Recombinant human TGF- $\beta$ 2 (R & D Systems,  
639 Minneapolis, MN) was added to the culture medium at a concentration of 10 ng/ml and re-  
640 combinant VEGFA165 at a concentration of (5ng/ml, 50ng/ml) for all relative experiments.  
641 NFAT inhibitor (VIVIT peptide) (EMD Biosciences, Darmstadt, Germany), was added to  
642 the culture medium at a concentration of 10  $\mu$ M for all relative experiments. Cells were  
643 passaged 1:3 or 1:4 every 3-6 d and used between passages 4 and 8.

644 *RT-PCR* RNA extractions were performed using a Qiagen total RNA purification kit (Qi-  
645 agen, Valencia, CA) and RNA was reverse transcribed to cDNA using the SuperScript  
646 III RT-PCR kit with oligo(dT) primer (Invitrogen). Sufficient quality RNA was determined  
647 by an absorbance ratio A260/A280 of 1.8-2.1, while the quantity of RNA was determined  
648 by measuring the absorbance at 260nm (A260). Real-time PCR experiments were con-  
649 ducted using the SYBR Green PCR system (Biorad, Hercules, CA) on a Biorad CFX96  
650 cycler, with 40 cycles per sample. Cycling temperatures were as follows: denaturing,  
651 95C; annealing, 60C; and extension, 70C. Primers were designed to detect GAPDH, E-  
652 cadherin, vimentin, Slug, Sp1, and NFATc1 in cDNA clones: Sp1 (F-TTG AAA AAG GAG  
653 TTG GTG GC, R-TGC TGG TTC TGT AAG TTG GG, Accession NG030361.1), NFATc1  
654 (F-GCA TCA CAG GGA AGA CCG TGT C, R-GAA GTT CAA TGT CGG AGT TTC TGA  
655 G, Accession NG029226.1). GAPDH, E-cadherin, vimentin, and Slug primers were taken  
656 from previously published literature Medici *et al.* (2008).

657 *Antibody Staining* Samples were fixed in 4% PFA overnight at 4C. Samples were then  
658 washed for 15 minutes on a rocker 3 times with PBS, permeabilized with 0.2% Triton-X  
659 100 (VWR International, Radnor, PA) for 10 minutes, and washed another 3 times with  
660 PBS. Samples were incubated overnight at 4C in a 1% BSA (Rockland Immunochemi-  
661 cals, Inc., Gilbertsville, PA) blocking solution followed by another 4C overnight incubation  
662 with either rabbit anti-human E-cadherin 1:100 (Abcam, ab53033), mouse anti-human  
663 phospho-Sp1 1:100 (Abcam, ab37707), mouse anti-human vimentin 1:100 (Invitrogen,  
664 V9), and rabbit anti-human NFATc1 (Santa Cruz, sc-7294) 1:100. After 3 washes for 15  
665 minutes with PBS, samples were exposed to Alexa Fluor 488 or 568 conjugated (Invit-  
666 rogen), species specific secondary antibodies at 1:100 in 1% BSA for 2 hours at room  
667 temperature. Three more washes with PBS for 15 minutes were followed by incubation  
668 with either DRAQ5 far red nuclear stain (Enzo Life Sciences, Plymouth Meeting, PA) at  
669 1:1000.

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

680 *Three-Dimensional Culture and Tubulogenesis Assays* For invasion/migration assays,  
681 cells were resuspended in culture media, and allowed to aggregate overnight in hanging  
682 drop culture (20 $\mu$ L; 20,000 cells). The spherical aggregates were placed on the surface of  
683 neutralized type I collagen hydrogels (1.5mg/mL) and allowed to adhere for 2 hrs before  
684 adding treatments. Cultures were maintained for 72 hrs, after which they were fixed in  
685 4% PFA and slowly rehydrated using PBS. For compaction assays, cells were pelleted  
686 via centrifugation and resuspended within a neutralized collagen hydrogel (1.5mg/mL)  
687 solution at a density of 400,000 cells/mL. 250 $\mu$ L of gel was inoculated into culture wells,  
688 which solidified after 60min. Treatments were then added within 800 $\mu$ L of the culture  
689 medium without serum. Gels were liberated from the surfaces of the culture wells the  
690 next day and cultured free floating for an additional 3-7 days, exchanging serum free  
691 media with appropriate factors every 48 hrs.

692 Tubulogenesis was defined as a typical nonmalignant acini structure. This includes  
693 a polarized epithelial cell, hollow lumen, and the basal sides of the cell are surrounded  
694 by ECM proteins (Fig. 6A, Controls or VEGF treated). Previous work has shown that  
695 change in the morphological characteristics of nontumorigenic MCF10A epithelial acini  
696 occur over time and exploiting them to growth in 3D culture can be quantified. For exam-

697 ple, using image segmentation, Chang et al. Chang *et al.* (2007) examined the elongation  
698 of the MCF10A acini at 6, 12, and 96 hours after a particular treatment. Polizzotti et al.  
699 Polizzotti *et al.* (2012) also suggested a computational method to quantify acini structure  
700 based on morphological characteristics in nonmalignant, noninvasive, and invasive con-  
701 ditions. Adapted from these approaches, we first fluorescently labeled our cultures and  
702 captured the acini structures by 3D confocal microscopy. Next individual acini structures  
703 in the images were segmented by imageJ and labeled. We then extracted the number of  
704 ductal branches. Ductal branching was defined as any elongated cell cluster extending  
705 away from the total acini structure, which was manually segmented and counted using Im-  
706 ageJ. A total of 5 images for each condition were used, and approximately 12 acini were  
707 analyzed in each image. Total branching was normalized to the amount of acini present,  
708 and provides an overall general assessment to the extent of acini remodeling.

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

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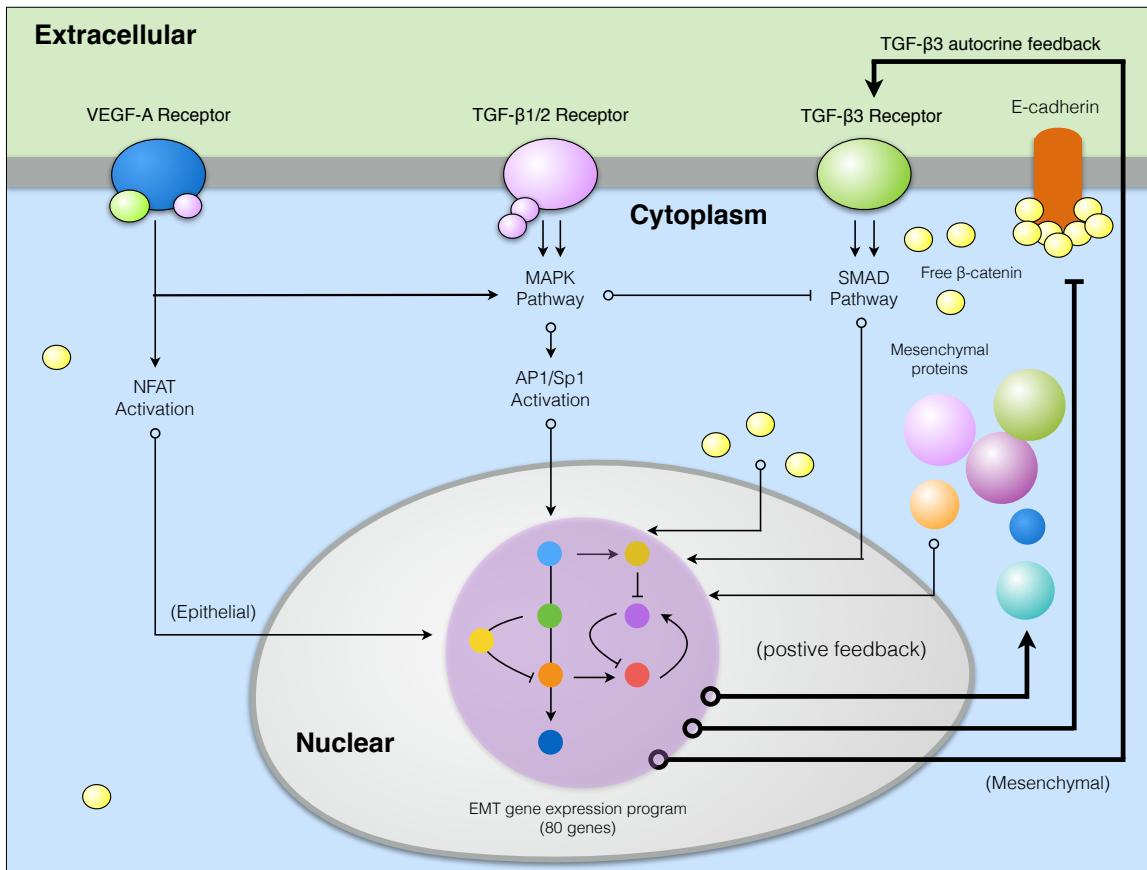
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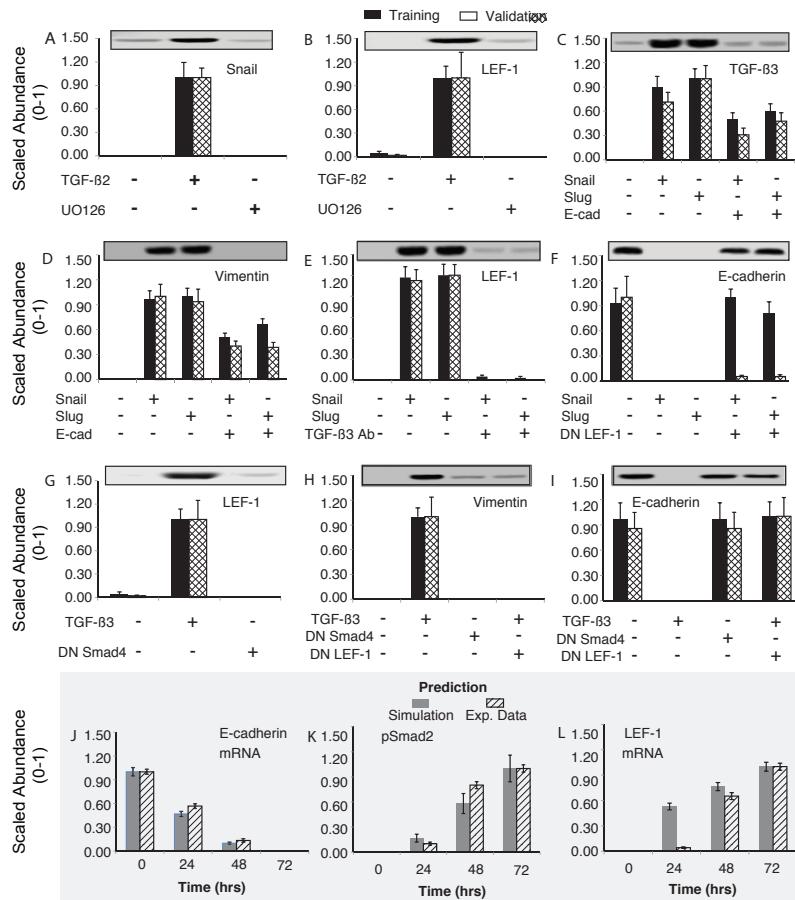
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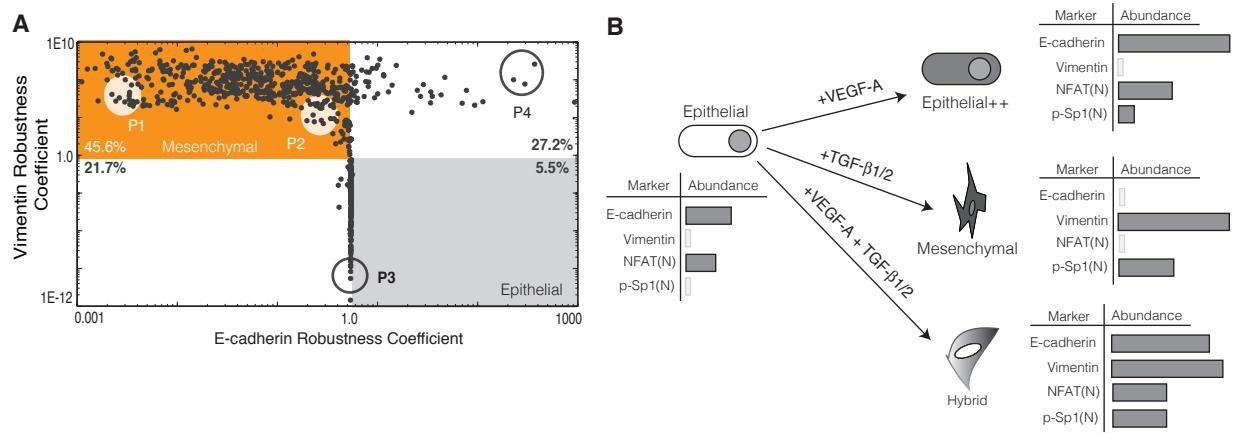
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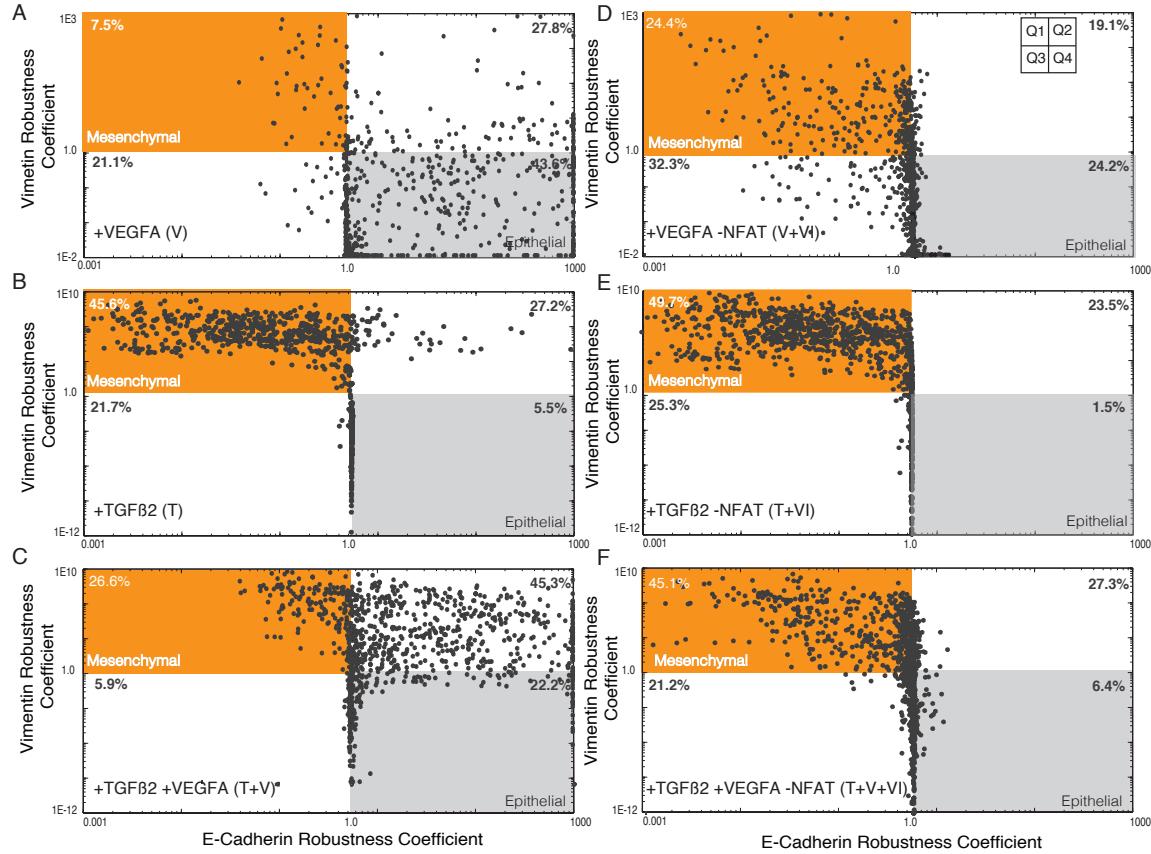
**Fig. 1:** Model connectivity recreates the core architecture during EMT. The EMT network contains 995 nodes (proteins, mRNA, and genes) interconnected by 1700 interactions. Central to EMT induction, activation of the MAPK cascade occurs through TGF- $\beta$ 1/2 binding which activates the AP-1/Sp1 transcriptional axis. AP-1/Sp1 drives an autocrine response of TGF- $\beta$ 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.



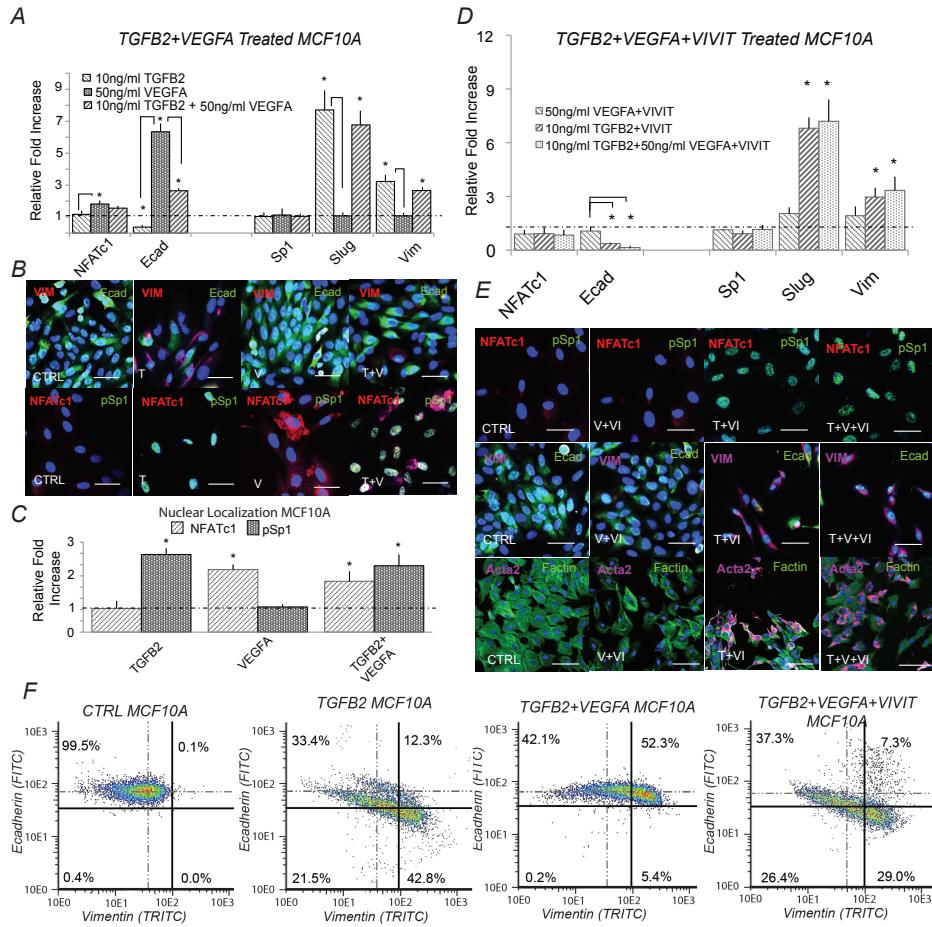
**Fig. 2:** Training and validation simulations. The population of EMT models qualitatively captured TGF $\beta$ -induced EMT signaling. (A-I) The population was generated using POETs and trained using 11 different objective functions (41 data sets) taken from Medici *et al.* Medici *et al.* (2008). 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.



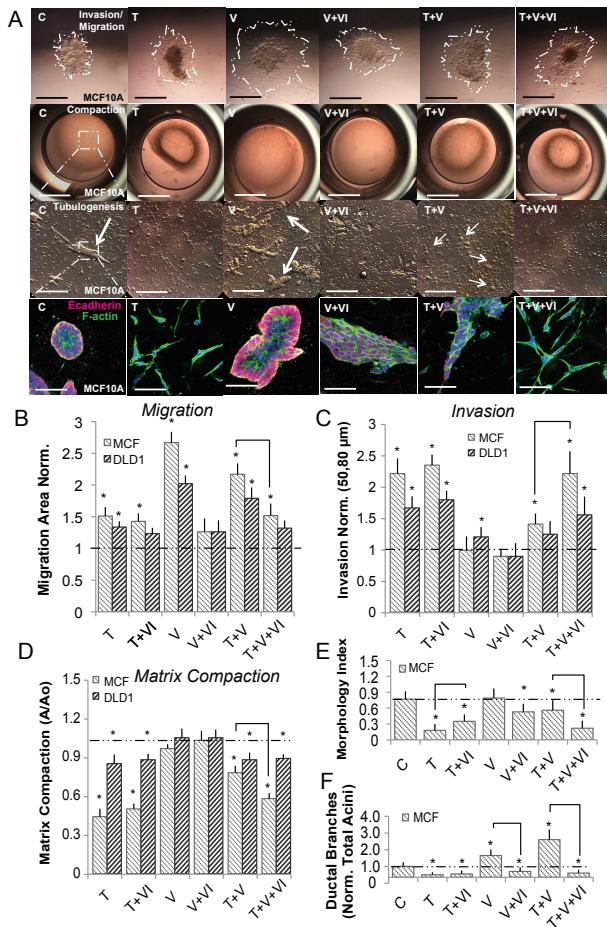
**Fig. 3:** Simulated TGF $\beta$ 1/2 exposure promoted phenotype heterogeneity. Robustness coefficients were used to quantify the effect of perturbations after steady state across the population of data sets. Coefficients with values  $> 1$  ( $< 1$ ) indicated a marker increased (decreased) compared 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 $\beta$ 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 $\beta$ 2 and VEGFA pathway, respectively. (C) We hypothesized that elevated phosphorylated-Sp1 and NFAT levels could drive phenotype heterogeneity through simultaneous TGF $\beta$ 2 and VEGFA treatment, similar to region three (R3).



**Fig. 4:** Simulated response to TGF $\beta$ 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 $\beta$ 2 (10 a.u.), which shifted the population towards a mesenchymal phenotype (Q1). Interestingly, the combined effects of TGF $\beta$ 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 $\beta$  mitigated all ecadherin expression (Q2) confirming their importance for population heterogeneity. Lastly, combination of TGF $\beta$ 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.



**Fig. 5:** Simultaneous TGF- $\beta$ 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 $\beta$ 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 $\beta$ 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 $\beta$ 2 and VEGFA, respectively. (D) To isolate the effect of NFAT, treatment of VEGFA (50ng/ml) and VIVIT (10 $\mu$ M) reduced ecadherin expression at 48hrs (control-dashed line). Similarly, combined TGF $\beta$ 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 $\mu$ m. C=Control, T=TGF $\beta$ 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 ( $p < 0.05$ ).



**Fig. 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 $\beta$ 2 (10ng/ml) enhanced invasion and contractile properties while, VEGFA (50ng/ml) promoted increased migration. TGF $\beta$ 2 with VEGFA significantly increased migration, while limiting with compaction. VIVIT (10 $\mu$ M) in combination with VEGFA and TGF $\beta$ 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 $\beta$ 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 $\mu$ m, 1000 $\mu$ m, 250 $\mu$ m, and 80 $\mu$ m, respectively. C=Control, T=TGF $\beta$ 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.

1038 **Supplemental Materials and Methods**

1039 **EMT model network architecture.** TGF $\beta$  is a major inducer of EMT in development, fi-  
1040 brosis, and carcinogenesis with different isoforms mediating various effects depending on  
1041 specific cellular context Nawshad *et al.* (2005). TGF $\beta$ 1 was first described as an inducer  
1042 of EMT in normal mammary epithelial cells Miettinen *et al.* (1994) and has since been  
1043 shown to mediate EMT in vitro in a number of different epithelial cells, including renal  
1044 proximal tubular, DLD1 colon carcinoma, and most recently alveolar epithelial cells Fan  
1045 *et al.* (1999), Hales *et al.* (1994), Kasai *et al.* (2005), Willis *et al.* (2005). TGF $\beta$  signaling  
1046 occurs through the Smad pathway in which signals are transduced by transmembrane  
1047 serine/threonine kinase type I (ALK5) and type II (TGF $\beta$ RII) receptors. To increase lig-  
1048 and affinity, betaglycan (TGF $\beta$ RIII) can also interact with TGF $\beta$ RI,II Gatza *et al.* (2010).  
1049 Upon TGF $\beta$  stimulation, the receptors are internalized into early endosomes where Smad  
1050 anchor for receptor activation (SARA) modulates formation of complexes with (R-Smad)  
1051 Smad2 or Smad3. Smad2 and Smad3 are then phosphorylated at serine residues by  
1052 the type I receptor Massagué *et al.* (2005). Phosphorylation induces their association  
1053 with (Co-Smad) Smad4 and translocation to the nucleus where they interact with other  
1054 transcription factors to regulate the transcription of TGF $\beta$  responsive genes, including  
1055 alpha-smooth muscle actin, collagen1A2, vimentin, fibronectin, and plasminogen activa-  
1056 tor inhibitor-1 (PAI-1) by interacting with Smad-binding elements Dennler *et al.* (2002),  
1057 Derynck & Zhang (2003). To regulate TGF $\beta$  signaling, Smurf2 (a ubiquitin E3 ligase) can  
1058 become activated to mediate proteasome dependent degradation of Smad2 or bind with  
1059 Smad 7 to target TGF $\beta$  receptor for degradation Bonni *et al.* (2001), Kavsak *et al.* (2000).  
1060 **Cell Type Dependency** Interestingly, differential roles for Smad2 and Smad3 in TGF $\beta$   
1061 induced EMT have been demonstrated. For example, using primary cells from mice  
1062 with hepatocyte-specific double knockout of Smad2 and Smad3, it was demonstrated  
1063 that Smad3 but not Smad2 was required for a key morphological changes and induc-  
1064 tion of EMT Ju *et al.* (2006). 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 Phanish *et al.* (2006). Furthermore, Smad2 signaling has been demonstrated in cancerous lung epithelial A549 cells Kasai *et al.* (2005). Inhibition of Smad3 signaling decreases the metastatic potential of xenografted breast cancer cell lines Tian *et al.* (2004, 2003). Together, these results suggest that the precise Smad pathway activated may depend on the particular cellular context. Regardless, a recent transcriptomic analysis of TGF $\beta$  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 Valcourt *et al.* (2005).

*Smad-Independent EMT Induction Pathways.* Although less well established than the Smad-dependent pathways in the induction of EMT, there is substantial evidence for TGF $\beta$  activation of 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 $\beta$  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 (LL-CPK1) cells Masszi *et al.* (2003). TGF $\beta$  has been shown to elevate ERK activity in culture models of EMT (human keratinocytes, NMuMG mammary epithelial cells, and mouse cortical tubule epithelial cells) Davies *et al.* (2005), Xie *et al.* (2004), Zavadil *et al.* (2001). This ERK activity was required for disassembly of adherens junctions and induction of cell motility Xie *et al.* (2004). Inhibition of ERK blocked these key morphological changes of EMT in mammary gland epithelial cells Xie *et al.* (2003). Recent studies in MDCKII and DLD1 colon carcinoma cells revealed that TGF $\beta$ 3 can signal through PI3 kinase. Upon activation, PI3 kinase activates molecules such as ILK and AKT, which can phosphory-

1092 late and inactivate GSK3 $\beta$ , a protein that targets both Snail and  $\beta$ -catenin for degradation  
1093 through the ubiquitin proteosome pathway Medici *et al.* (2008). Lastly, there is increasing  
1094 evidence for a role of Notch pathways in regulating EMT, particularly during development  
1095 Nawshad *et al.* (2005). In cardiac cushion EMT, it was found that that Slug is directly  
1096 up-regulated by Notch in endothelial cells repression of the vascular endothelial cadherin  
1097 promoter and for promoting migration of transformed endothelial cells. In whole, the dis-  
1098 tinction between Smad-dependent and Smad-independent mechanisms remains difficult  
1099 to interpret due to the significant cross talk between these pathways. In most cases, stim-  
1100 ulation of these cooperative pathways provides the context for induction and specification  
1101 of EMT within a particular tissue/cell type, with Smads representing the dominant path-  
1102 way, which in some instances may be necessary but not sufficient for induction of full EMT  
1103 Zavadil & Böttinger (2005).

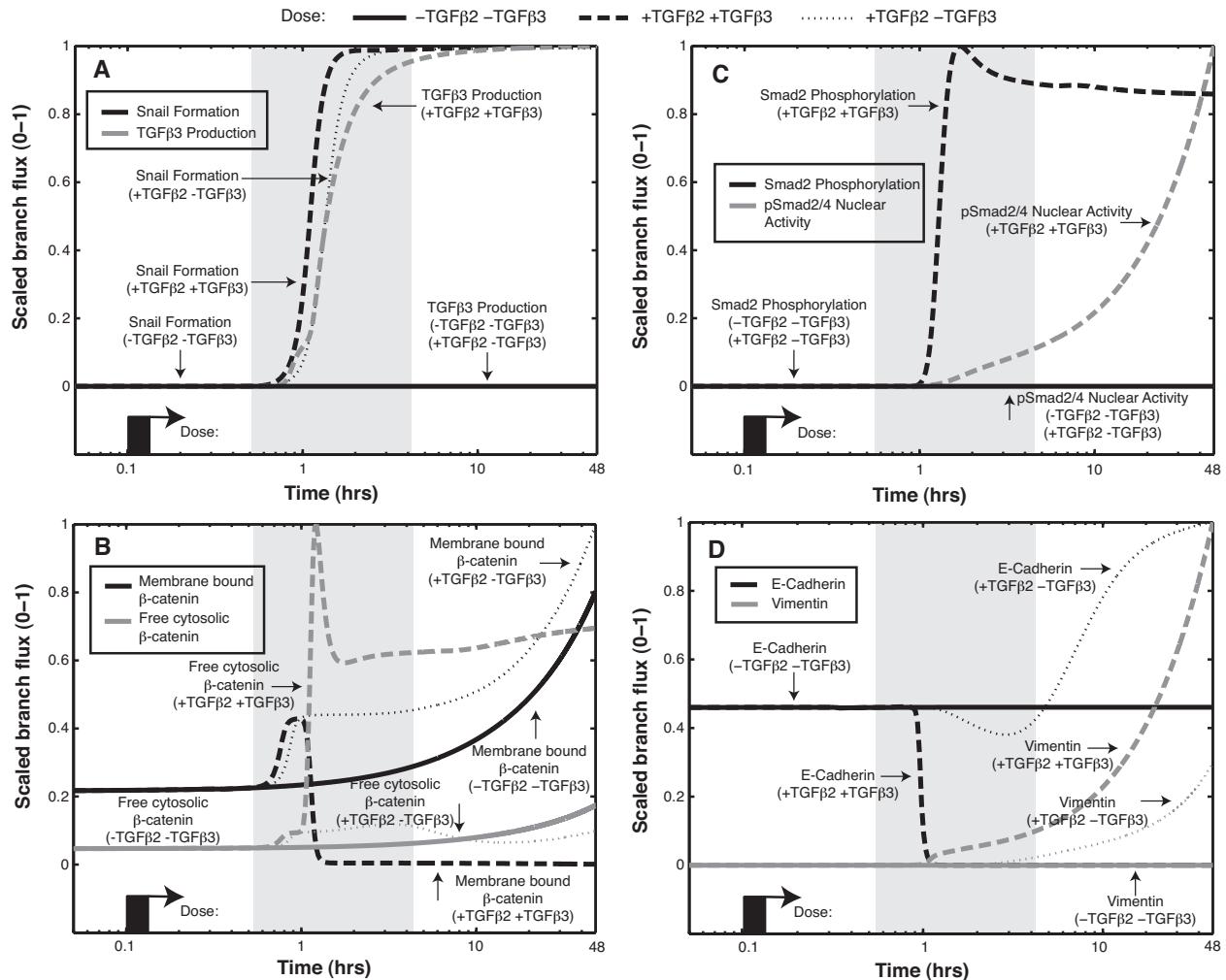
1104 *Master Transcription Regulators* Master transcription factors of EMT are thought to be  
1105 regulated by Snail1 (SNAI1) and Snail2 (SNAI2) (previously known as Snail and Slug,  
1106 respectively). These transcription factors are zinc finger proteins that function as repres-  
1107 sors of E-cadherin transcription in cultured epithelial cells Cano *et al.* (2000), repression  
1108 of E-cadherin leads to dissolution of adherens junctions. Snail and Slug can be activated  
1109 by TGF $\beta$  via both Smad-dependent and -independent pathways in a cell type dependent  
1110 fashion in cultured cells Peinado *et al.* (2003). Differential expression of Snail and Slug  
1111 is observed in TGF $\beta$  induced EMT in keratinocytes, renal proximal tubular, and mam-  
1112 mary epithelial cells, suggesting that they are regulated in a cell specific. Interestingly,  
1113 key regulatory units of Snail and Slug appear to be regulated by upstream transcription  
1114 factors AP1 and SP1, respectively. Peinado et al. clearly established a necessary role  
1115 of Snail in mediating TGF $\beta$ 1 induced EMT in MDCK cells. They demonstrated that the  
1116 Ras-Raf-MEK-ERK-AP1 signaling pathway could up-regulate synthesis of the E-cadherin  
1117 repressor molecule Snail (directly binds to Snail promoter) Peinado *et al.* (2004). Like-  
1118 wise, TGF $\beta$ 1 induced the expression of Slug in both lens and other epithelial cells in vitro.

1119 The Sp1 binding site in the Slug promoter is largely responsible for TGF $\beta$ 1 induced Slug  
1120 expression and upstream of MAPK signaling. In addition, the TGF $\beta$ 1 mediated repression  
1121 of E-cadherin was significantly inhibited by Slug siRNA Choi *et al.* (2007).

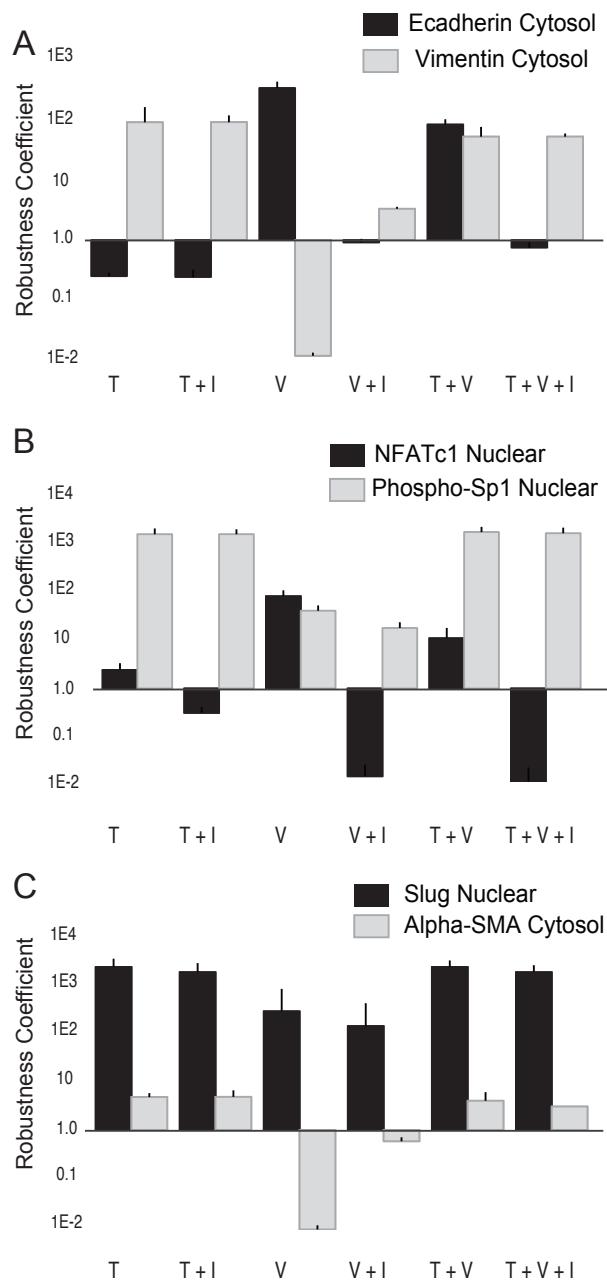
1122 **ERK/MAPK Crosstalk** The ERK-MAPK pathway has been shown to modify TGF $\beta$  sig-  
1123 naling at multiple levels. One way is that the activated Ras pathway inhibits the TGF $\beta$   
1124 induced nuclear accumulation of Smad2/3, as seen in epithelial cells Zhang (2009). ERK  
1125 kinases have been shown to phosphorylate Smad2 and Smad3 at specific sites in the re-  
1126 gion linking the MH1 and MH2 domains. These sites are separate from the TGF $\beta$  receptor  
1127 phosphorylation sites which activate Smad nuclear translocation Massague (2003). The  
1128 effect of interaction between ERKs and Smads is the subject of some controversy, with  
1129 data suggesting that such an interaction either enhances or inhibits downstream events.  
1130 A hyperactive Ras pathway has been shown to effectively counteract the antiproliferative  
1131 activity of TGF $\beta$  through attenuation of Smad accumulation in the nucleus Kretzschmar  
1132 *et al.* (1999). In contrast, Ras signals strongly cooperated with Smads for invasion of  
1133 human carcinoma cells Oft *et al.* (1996). A second way is through transcriptional regula-  
1134 tion. Recently, it was found that Sp1, via transcriptional induction of Vimentin, cooperates  
1135 with activated Smad complexes in mesenchymal transition and migration of pancreatic  
1136 cancer cells upon TGF $\beta$  stimulation Jungert *et al.* (2007). Likewise, binding sites at the  
1137 SP1/Smad3 complex was found to regulate the betaglycan receptors Lopez-Casillas *et al.*  
1138 (2003). Taken together, multiple levels of cross-talk (both positive and negative) exist  
1139 within MAPK and TGF $\beta$ , and may also be cell type dependent.

1140 **Analysis of the signal flow through the EMT architecture.** To investigate temporal  
1141 shifts for key species dominating the EMT response, we calculated the scaled flux through  
1142 the signaling architecture (Fig. S1). Three modes of operation were simulated to iden-  
1143 tify distinct behavioral differences: (a) no TGF- $\beta$ 2 (-TGF- $\beta$ 1/2), (b) TGF- $\beta$ 1/2 stimulation  
1144 (+TGF- $\beta$ 2), and (c) TGF- $\beta$ 1/2 stimulation while blocking the autocrine response of TGF-  
1145  $\beta$ 3 (+TGF- $\beta$ 1/2 + TGF- $\beta$ 3Ab). Following TGF- $\beta$ 1/2 stimulation, AP1/SP1 rapidly stimu-

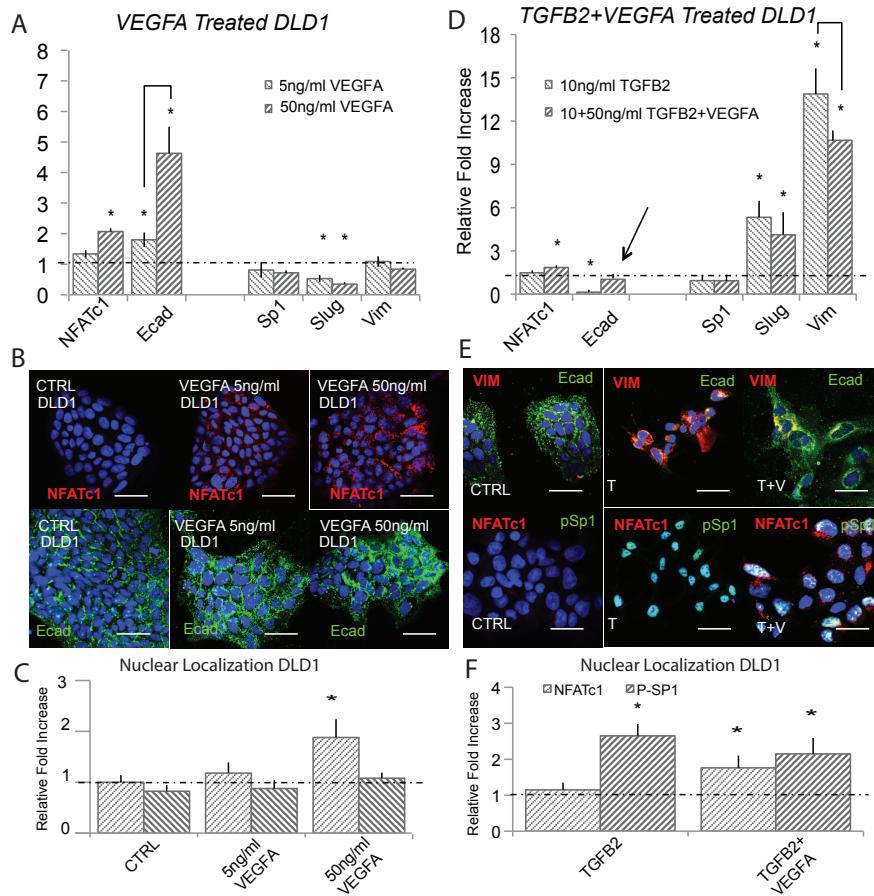
1146 lates Snail/Slug expression (Fig. S1A) within 1 hr. During this phase, Snail/Slug repress  
1147 E-cadherin expression, which in turn reduces the interaction between E-cadherin and  
1148  $\beta$ -catenin, and the accumulation of free cytosolic  $\beta$ -catenin ensues. Conversely, in the  
1149 presences of a TGF- $\beta$ 3 neutralizing antibody, membrane bound  $\beta$ -catenin increased af-  
1150 ter 10 hrs, while low levels of free cytosolic  $\beta$ -catenin accumulated between 1-5 hrs (Fig.  
1151 S2A,B). Accumulation of free  $\beta$ -catenin is critical for complexing with TCF4 to rapidly  
1152 produce the autocrine response of TGF- $\beta$ 3 within 1-10 hrs. Following TGF- $\beta$ 3 expres-  
1153 sion, formation and spatial relocation of the Smad complexes occurs in a time dependent  
1154 manner. Smad2 is phosphorylated within 1 hr and nuclear localization of the pSmad2/4  
1155 complex dramatically increases after 10 hours (Fig. S1C). This timely formation is crit-  
1156 ical for completion of the EMT process. At steady state, the E-cadherin complex was  
1157 maintained at a basal epithelial level with no expression of Vimentin (Fig. S1D). During  
1158 TGF- $\beta$ 1/2 stimulation, both MAPK and Smad act synergistically to repress the E-cadherin  
1159 complex within 1 hr. This is followed by increase of Vimentin at  $\sim$  1hr, while exponentially  
1160 increasing around 10 hrs. Both species elevate within 1 hr, however the complex does not  
1161 significantly form until  $\sim$  10 hrs. When blocking TGF- $\beta$ 3, Snail/Slug downregulates the  
1162 E-cadherin complex between 5-10 hrs, but cannot complete the transformation due to the  
1163 loss of Smad2 phosphorylation (Fig. S1D).



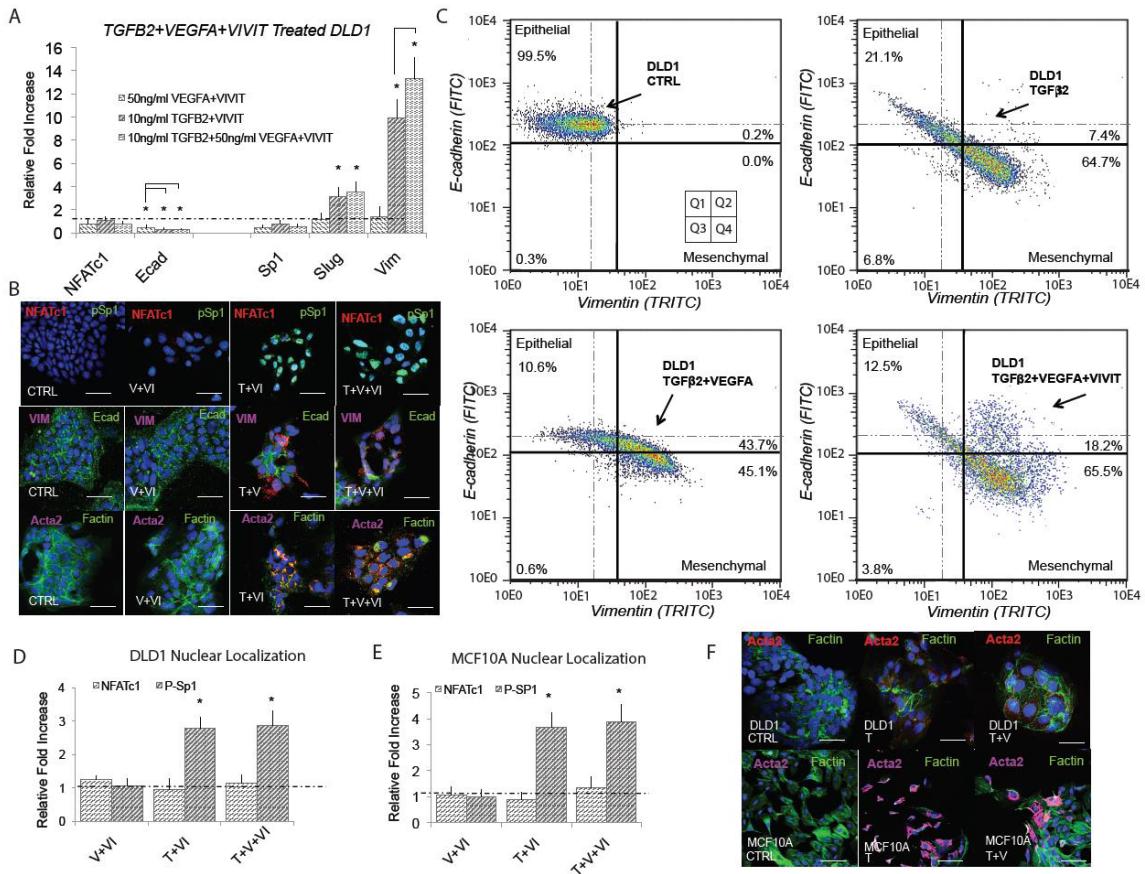
**Fig. S1:** Signal flow analysis of key species at steady state, TGF- $\beta$ 1/2 stimulation, and blocking the TGF- $\beta$ 3 autocrine response. (A) The MAPK cascade is directly responsible for rapid expression of Snail and downstream TGF- $\beta$ 3 formation (1 hrs). (B) TGF- $\beta$ 1/2 reduces  $\beta$ -catenin, allowing rapid free-cytosolic  $\beta$ -catenin to accumulate (1hr). Blocking TGF $\beta$ 3 increases membrane bound  $\beta$ -catenin (10hr). (C) TGF- $\beta$ 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- $\beta$ 1/2 rapidly reduces the E-cadherin complex, while upregulating Vimentin (5-10 hrs). Blocking TGF $\beta$ 3 increases E-cadherin (10 hrs) and Vimentin is significantly reduced.



**Fig. S2:** Robustness analysis for key molecular species at  $t = 48$  hrs for combinations of  $\text{TGF-}\beta 1/2$ ,  $\text{VEGF-A}$  and  $\text{NFATc1}$  inhibitors. Robustness coefficients for the indicated species were calculated for  $N \sim 1100$  ensemble members for 48 hrs following the addition of  $\text{TGF-}\beta 1/2$  (T),  $\text{TGF-}\beta 1/2 + \text{NFATc1 inhibitor}$  (T + I),  $\text{VEGF-A}$  (V),  $\text{VEGF-A} + \text{NFATc1 inhibitor}$  (V + I) and  $\text{TGF-}\beta 1/2 + \text{VEGF-A}$  (T+V) +  $\text{NFATc1 inhibitor}$  (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  $\alpha$ -smooth muscle actin ( $\alpha$ -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).



**Fig. S3:** VEGF-A attenuates TGF- $\beta$ 1/2 to induce phenotype heterogeneity in DLD1. (A) In DLD1, we found that 5ng/ml of VEGF-A 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 $\beta$ 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 VEGF-A (50ng/ml) and TGF $\beta$ 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 $\mu$ m. C=Control, T=TGF $\beta$ 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 ( $p < 0.05$ ).



**Fig. 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 $\beta$ 2 (10ng/ml) and VIVIT resulted in increased Slug and vimentin expression, while inhibiting ecadherin levels. Combined TGF $\beta$ 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 $\beta$  conditions. (C) Quantitative flow cytometry also confirmed this trend. (D,E) TGF $\beta$ 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 $\beta$  conditions. Magnification, 40x. Scale bars: 50 $\mu$ m. C=Control, T=TGF $\beta$ 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 ( $p < 0.05$ ).

1164 **Estimation and cross-validation of EMT model parameters.** We used the Pareto  
 1165 Optimal Ensemble Technique (POETs) multiobjective optimization framework in combi-  
 1166 nation with leave-one-out cross-validation to estimate an ensemble of TGF $\beta$ /EMT mod-  
 1167 els. Cross-validation was used to calculate both training and prediction error during the  
 1168 parameter estimation procedure Kohavi (1995). The 41 intracellular protein and mRNA  
 1169 data-sets used for identification were organized into 11 objective functions. These 11  
 1170 objective functions were then partitioned, where each partition contained ten training ob-  
 1171 jectives and one validation objective. POETs integrates standard search strategies e.g.,  
 1172 Simulated Annealing (SA) or Pattern Search (PS) with a Pareto-rank fitness assignment  
 1173 Song *et al.* (2010). Denote a candidate parameter set at iteration  $i + 1$  as  $\mathbf{k}_{i+1}$ . The  
 1174 squared error for  $\mathbf{k}_{i+1}$  for training set  $j$  was defined as:

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

1175 The symbol  $\hat{\mathcal{M}}_{ij}$  denotes scaled experimental observations (from training set  $j$ ) while  
 1176  $\hat{y}_{ij}$  denotes the scaled simulation output (from training set  $j$ ). The quantity  $i$  denotes  
 1177 the sampled time-index and  $\mathcal{T}_j$  denotes the number of time points for experiment  $j$ . In  
 1178 this study, the experimental data used for model training was typically the band intensity  
 1179 from Western or Northern blots. Band intensity was estimated using the ImageJ software  
 1180 package Abramoff *et al.* (2004). The scaled measurement for species  $x$  at time  $i =$   
 1181  $\{t_1, t_2, \dots, t_n\}$  in condition  $j$  is given by:

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

1182 Under this scaling, the lowest intensity band equaled zero while the highest intensity band  
 1183 equaled one. A similar scaling was defined for the simulation output. By doing this scal-  
 1184 ing, we trained the model on the relative change in blot intensity, over conditions or time  
 1185 (depending upon the experiment). Thus, when using multiple data sets (possibly from

1186 different sources) that were qualitatively similar but quantitatively different e.g., slightly  
1187 different blot intensities over time or condition, we captured the underlying trends in the  
1188 scaled data. Additionally, we applied a universal convention of identifying the no expres-  
1189 sion case as protein value below  $<10^{-3}$ . This is similar to previously published models  
1190 from our lab. In our studies, Figure 2 identifies experimental data extracted from pub-  
1191 lished Western blots and our simulation results. It is clear from these that 1) the training  
1192 data included a variety of Western blot data treatments that were effectively zero, and 2)  
1193 our simulations matched the training data virtually perfectly over time and across multi-  
1194 ple biological species. These results validate the power of our simulation scheme, which  
1195 necessarily includes numerical interpretations of zero. In our Supplemental Figure S9, we  
1196 present the raw concentrations of our simulations (previously validated from the training  
1197 data) over hundreds of parameter ensembles, with no “zero” threshold applied. While  
1198 these values do not have a threshold, we only interpret values below  $10^{-3}$  equivalent to  
1199 zero. Likewise, our robustness coefficients (Figure 3) (which represent the ratio of in-  
1200 tegrated areas of the treatment effect over the baseline effect) identify no difference in  
1201 model output for vimentin (or e-cadherin for that matter) less than  $10^{-3}$ , confirming this  
1202 interpretation.

1203 We computed the Pareto rank of  $\mathbf{k}_{i+1}$  by comparing the simulation error at iteration  
1204  $i + 1$  against an archive of accepted parameter sets  $\mathbf{K}_i$ . We used the Fonseca and  
1205 Fleming ranking scheme Fonseca *et al.* (1993) to estimate the number of parameter sets  
1206 in the archive that dominate  $\mathbf{k}_{i+1}$ . Parameter sets with increasing rank were progressively  
1207 further away from the optimal trade-off surface. The parameter set  $\mathbf{k}_{i+1}$  was accepted or  
1208 rejected by POETs with probability  $\mathcal{P}(\mathbf{k}_{i+1})$ :

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

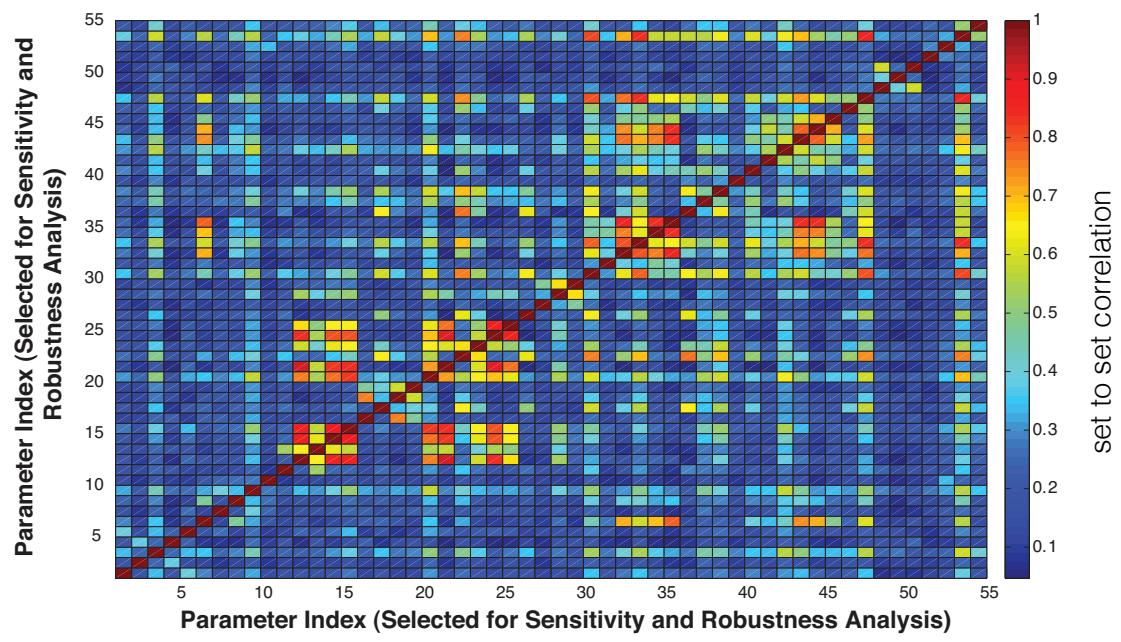
1209 where  $T$  is the annealing temperature and  $rank(\mathbf{k}_{i+1} | \mathbf{K}_i)$  denotes the Pareto rank for  
1210  $\mathbf{k}_{i+1}$ . The annealing temperature was discretized into 10 quanta between  $T_o$  and  $T_f$  and

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	TGFbeta3	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	"

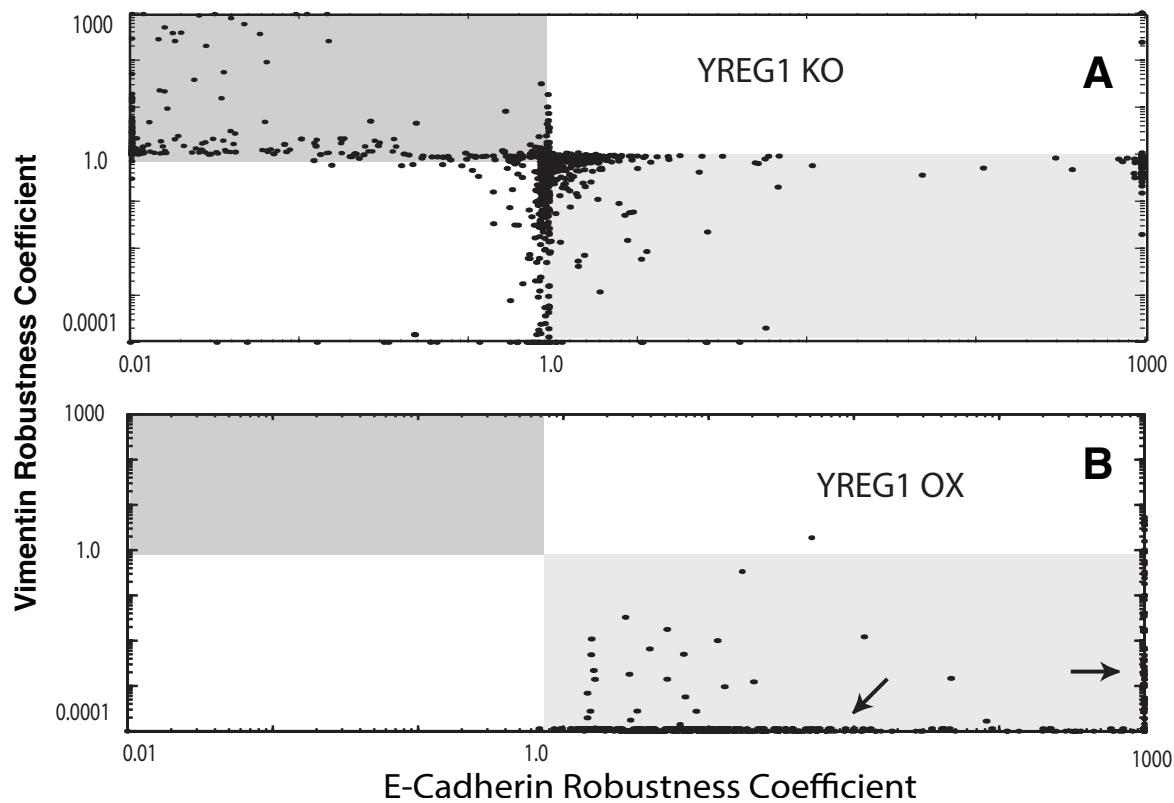
**Fig. S5:** Training and prediction values for the 11 TGF- $\beta$  objective functions versus a random parameter control.

adjusted according to the schedule  $T_k = \beta^k T_0$  where  $\beta$  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 Gadkar et al. (2003). From the 15,000 probable EMT models, we selected  $N = 1093$  models with Pareto rank  $\leq 1$  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.

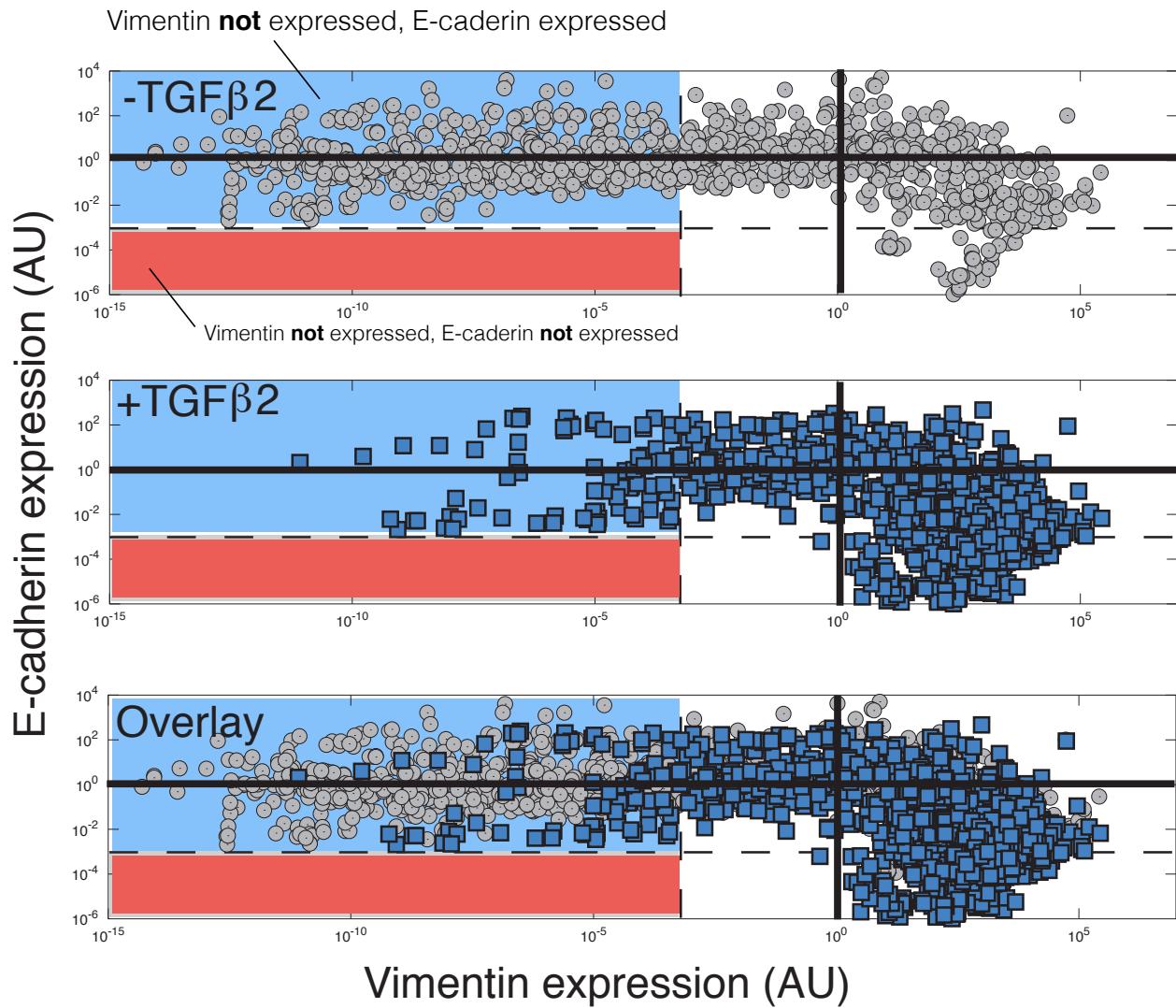
**Fig. S6:** Training and prediction values as a function of condition for the 11 TGF- $\beta$  objective functions versus a random parameter control.



**Fig. 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.



**Fig. 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.



**Fig. S9:** Steady state protein abundance for E-cadherin and Vimentin (AU) as a function of TGF- $\beta$ 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- $\beta$ 1/2. Midddle: Vimentin (AU) and E-cadherin (AU) expression in the absence of TGF- $\beta$ 1/2 showed exhibited population heterogeneity. Bottom: Vimentin (AU) and E-cadherin (AU) expression in the presence of TGF- $\beta$ 1/2 moved the centriod of the population toward Vimentin (AU) and away from E-cadherin (AU) expression.