

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

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Abstract

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

Author Summary

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

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 β (TGF- β) 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- β isoforms induce
15 EMT across a variety of cell lines (Medici *et al.*, 2006, 2008). This program involves
16 carefully orchestrated rounds of gene expression driven by the Smad and Snail families
17 of transcription factors as well as other key factors such as lymphoid enhancer-binding
18 factor 1 (LEF-1), nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), and speci-
19 fity protein 1 (Sp1). Coregulators such as β -catenin, NF- κ B, and the ErbB family of
20 receptor tyrosine kinases however also participate in EMT regulation, but the degree of
21 each's influence is difficult to ascertain in isolation (Hardy *et al.*, 2010, Huber *et al.*, 2004,
22 Jiang *et al.*, 2007, Kim *et al.*, 2002). EMT also exhibits complex temporal dynamics that
23 are 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- β induced differentiation focused on single biological factors or
29 EMT in single cells. For example, Chung *et al.*, constructed a model of TGF- β 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- β 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, includ-
36 ing competing second messengers and interconnected transcriptional regulatory loops.
37 Integrating these additional scales of molecular signaling while maintaining the capacity

38 for robust prediction requires a new and expanded computational and experimental strat-
39 egy. Data-driven systems approaches (Cirit & Haugh, 2012) or logical model formulations
40 (Morris *et al.*, 2011) are emerging paradigms that constrain model complexity through
41 the incorporation of training and validation data. These are interesting techniques be-
42 cause the data informs model structure (which can be expanded as more data becomes
43 available). Alternatively, Bailey proposed more than a decade ago that a qualitative un-
44 derstanding of a complex biological system should not require complete definition of its
45 structural and parametric content (Bailey, 2001). Shortly thereafter, Sethna and cowork-
46 ers showed that complex model behavior is often controlled by only a few parameter
47 combinations, a characteristic seemingly universal to multi-parameter models referred
48 to as “sloppiness” (Machta *et al.*, 2013). Thus, reasonable model predictions are often
49 possible with only limited parameter information. Taking advantage of this property, we
50 developed sloppy techniques for parameter identification using ensembles of determin-
51 istic models (Song *et al.*, 2010). Furthermore, we proposed that the sloppy behavior of
52 biological networks may also be seen as a source of cell-to-cell (Lequieu *et al.*, 2011) or
53 even patient-to-patient heterogeneity (Luan *et al.*, 2010). Recently, Bayesian parameter
54 identification techniques have also been used to explore cell-to-cell heterogeneity (Hase-
55 nauer *et al.*, 2011, Kalita *et al.*, 2011), where a population of cells could be viewed as a
56 dynamic ensemble of 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- β 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 97 gene,
61 protein or mRNA components interconnected through 169 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 (JuPOETs) multiobjective optimization algorithm. JuPOETs generated an ensemble of

65 approximately 1400 models for analysis. Analysis of the model population suggested that
66 both MCF10A and DLD1 cells could exhibit phenotypic heterogeneity if treated simultane-
67 ously with TGF- β 1/2 and VEGF-A. This heterogeneity was characterized by a significant
68 fraction of the population being in a “hybrid state” having both high E-cadherin and high
69 Vimentin expression. We tested these predictions using qRT-PCR and flow-cytometry
70 studies in a variety of experimental conditions. Validation studies confirmed that upwards
71 of 45% of the cellular population could be put into the hybrid state in the presence of both
72 TGF- β 1/2 and VEGF-A. Moreover, this response depended upon both activation of Sp1
73 by MAPK and NFATc1 transcriptional activity consistent with the predicted molecular sig-
74 naling. Lastly, the hybrid populations of both DLD1 and MCF10A cells exhibited different
75 functional behavior than those from either TGF- β or VEGF-A treatment. The extent of
76 ductal branch formation significantly increased with MCF10A cells in the hybrid pheno-
77 type, compared with cells treated with VEGF-A alone. Together, these results establish
78 a predictive mechanistic model of EMT susceptibility, and reveal a novel signaling axis,
79 which possibly regulates carcinoma progression through an EMT versus tubulogenesis
80 response.

81 **Results**

82 **The model population captured key features of TGF- β induced EMT** The EMT model
83 architecture, based upon curated molecular connectivity, described the expression of 23
84 genes following exposure to TGF- β isoforms and VEGF-A (Fig. 1). The EMT model con-
85 tained 74 molecular species interconnected by 169 interactions. Model equations were
86 formulated using either saturation or mass-action kinetics within an ordinary differential
87 equation (ODE) framework. ODEs and mass action kinetics are common tools to model
88 biochemical pathways (Chen *et al.*, 2009, Schoeberl *et al.*, 2002, Tasseff *et al.*, 2011).
89 However, while ODE models can simulate complex intracellular behavior, they require es-
90 timates for model parameters which are often difficult to obtain. The EMT model had 251
91 unknown model parameters, 169 kinetic constants and 45 non-zero initial conditions. As
92 expected, these parameters were not uniquely identifiable given the training data (Gad-
93 kar *et al.*, 2005). Thus, instead of identifying a single best fit (but uncertain) model, we
94 estimated a sloppy population of models (each consistent with the training data) by simul-
95 taneously minimizing the difference between model simulations and 41 molecular data
96 sets using the Pareto Optimal Ensemble Technique (JuPOETs). The training data were
97 generated in DLD1 colon carcinoma, MDCKII, and A375 melanoma cells following ex-
98 posure to TGF- β isoforms (Medici *et al.*, 2008). We organized these data sets into 11
99 objective functions which were simultaneously minimized by JuPOETs. Additionally, we
100 used 12 molecular data sets generated in HK-2 cells following VEGF-A exposure to train
101 VEGF-A responsive model processes (Lian *et al.*, 2011). To guard against overfitting,
102 we augmented the multiobjective optimization with leave-one-out cross validation to in-
103 dependently estimate both the training and prediction error for each objective. Thus, we
104 generated 11 different model ensembles. Lastly, we compared model predictions with in-
105 dependent data sets not used during training (both at the molecular and model population
106 levels) to evaluate the predictive power of the parameter ensemble.

107 JuPOETs generated a population of probable signaling models which captured the

multiple phases of EMT induction (Fig. 2). JuPOETs sampled well over 10^4 probable models during each stage of the cross-validation using global random sampling. From this analysis, $N \simeq 1400$ models were selected for further analysis. The selected models all had the same possible molecular connectivity, but different values for model parameters. Transcription and translation rates, as well as mRNA and protein degradation terms, were set using physical values from the literature (Milo *et al.*, 2010), and allowed to vary by a scaling factor, see methods. Model selection was based upon Pareto rank, the prediction and training error across all objectives. The model population recapitulated key signaling events following TGF- β exposure. We subdivided the response to TGF- β exposure into two phases. First, TGF- β 1/2 signaling initiated a program which downregulated E-cadherin expression in a MAPK dependent manner while simultaneously upregulating TGF- β 3 expression. Second, TGF- β 3 secretion initiated an autocrine feedback which upregulated the expression of mesenchymal markers such as Vimentin and key upstream transcription factors such as LEF-1 in a SMAD dependent manner. TGF- β 3 expression was also able to sustain β -catenin release by inhibiting its sequestration by the APC complex through PI3K mediated GSK3, which was captured by the model (Fig. 4B). Each phase involved the hierachal expression and/or post-translational modification of several key transcription factors. During the first phase, stimulation with TGF- β 1/2 (10 a.u.) activated both the SMAD and MAPK pathways. MAPK activation resulted in the phosphorylation of the transcription factor activator protein 1 (AP-1), which in-turn upregulated the expression of Snail, a well established transcriptional repressor (Fig. 2A). Snail expression was MAPK-dependent; the MEK inhibitor U0126 blocked AP-1 activation and Snail expression following TGF- β 1/2 exposure (Fig. 2A, Lane 3). Similar results were obtained for Slug expression, confirming initial activation through the MAPK pathway (data not shown). Overexpression of either Snail or Slug upregulated TGF- β 3 expression (Fig. 2C) while simultaneously downregulating E-cadherin expression (Fig. 2F). During the second phase, TGF- β 3 secretion and the subsequent autocrine signaling resulted in the

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

145 The predictive power of the ensemble was tested using both cross validation and by
146 comparing simulations with data sets not used for model training. In whole, 100% of our
147 training objectives were statistically significant (at a 95% confidence interval) compared
148 to a randomized parameter family ($N = 100$) generated from a random starting point.
149 Conversely, we *predicted* 100% of the training objectives, at a 95% confidence interval
150 compared to randomized parameters (Wicoxon non-parametric test). The model also cap-
151 tured the temporal gene expression responses of E-cadherin, pSmad2, and LEF-1 (not
152 used for model training) to within one-standard deviation (up to the 48 hr time-point) (Fig.
153 2J-L). Taken together, the model captured the key signaling events revealed by Medici *et*
154 *al.* (Medici *et al.*, 2008) that drive the phenotypic conversion. A listing of objective function
155 values resulting from training, cross validation and the random parameter control is given
156 in the supplement (Fig. S1).

157 **Identification of a novel LEF-1 regulator** During model identification, we found that
158 consistent TGF- β induced EMT from a stable epithelial cell population required an addi-
159 tional regulatory protein. This protein, which we called hypothetical regulator 1 (YREG1),
160 was required to mediate between SNAIL/SLUG transcriptional activity and the upregu-
161 lation of LEF-1 expression following TGF- β 1/2 exposure. SNAIL/SLUG are well known

transcriptional repressors (Dhasarathy *et al.*, 2011, Hemavathy *et al.*, 2000a,b), although there are a few studies which suggest that at least SNAIL can also act as a transcriptional activator (Guaita *et al.*, 2002). In the model, we assumed the expression of SNAIL/SLUG was likely regulated by AP1/SP1 (Jackstadt *et al.*, 2013). Thus, upon receiving direct SNAIL/SLUG and TGF- β 3 signals, the model predicted enhanced SNAIL/SLUG expression, consistent with experimental observations. TGF- β 1/2 stimulation also induces LEF-1 expression. However, literature evidence suggested that LEF-1 expression was not strongly dependent upon AP1/SP1 activity (Eastman & Grosschedl, 1999). Thus, either SNAIL/SLUG are acting as inducers (contrary to substantial biochemical evidence) or, they are repressing the expression of an intermediate repressor. Given the biochemical evidence supporting SNAIL/SLUG as repressors, we created the hypothetical YREG1 repressor whose expression is downregulated by SNAIL/SLUG. The literature data therefore suggested that YREG1 had two transcriptional targets, LEF-1 and TGF- β 3. By adding this regulator, our simulations became consistent with training and literature data. Medici *et al.* suggested that feedback between β -catenin and LEF-1 was likely, although this feedback had yet to be identified (Medici *et al.*, 2008). Low levels of YREG1 expression were present in all simulations to regulate the formation of the β -catenin-LEF-1 complex. To test the effect of YREG1 on the epithelial population, we conducted over-expression and knockdown simulations on untreated cells (Fig. 4C and 4D). In the absence of YREG1, the population of models failed to consistently retain a stable epithelial state (Fig. 4D). Conversely, YREG1 amplification revealed an enhanced epithelial phenotype, while some inherently transformed cells moved towards a hybrid phenotype (Fig. 4C). Elevated YREG1 repressed LEF-1 and TGF- β 3 expression, thereby not allowing free β -catenin to form the β -catenin-LEF-1 complex, or TGF- β 3 induced SMAD activation. Taken together, low YREG1 expression was required for the maintenance of a stable epithelial phenotype that was simultaneously inducible across TGF- β 1/2, TGF- β 3 and SNAIL/SLUG transfection, as seen in the training objectives.

189 TGF- β 1/2 and VEGF-A exposure promotes phenotype heterogeneity through NFATc

190 and phosphorylated Sp1 While we captured the central tendency of many of the molec-

191 ular features of EMT induction following TGF- β 1/2 exposure, an often neglected but im-

192 portant emergent feature of developmental and pathological programs is population het-

193 erogeneity (Park *et al.*, 2010). We (and others) have previously hypothesized that deter-

194 ministic model ensembles can simulate population behavior, at least at a course grained

195 level (Lequieu *et al.*, 2011). We tested this hypothesis by analyzing the response of the

196 population of EMT models to extracellular cues and then comparing this response to flow

197 cytometry studies. We quantified the phenotypic response of the individual members of

198 the ensemble to TGF- β 1/2 stimulation for two downstream phenotypic markers, Vimentin

199 (mesenchymal) and E-cadherin (epithelial) following the addition of TGF- β 1/2 alone (Fig.

200 3), and/or VEGF-A in combination with NFATc inhibitors (Fig. 3).

201 We identified model subpopulations that exhibited different behaviors following expo-

202 sure to TGF- β 1/2 (Fig. 3B). Analysis of the molecular signatures of these subpopulations

203 suggested the abundance, localization and state of the Sp1, AP-1 and NFATc transcription

204 factors controlled population heterogeneity. The majority of models (>80%) responded

205 to treatment, moving away from the untreated population (Fig 3A-F, gray). These mod-

206 els showed the classically expected behavior, a switch from an epithelial to mesenchy-

207 mal phenotype following TGF- β 1/2 exposure. Some models resembled untreated cells;

208 they had elevated phosphorylated Sp1, relative to non-induced cells, which decreased E-

209 cadherin expression through Slug-mediated inhibition, which in turn increased Vimentin

210 expression through TGF- β 3 autocrine signaling and the liberation of β -catenin. How-

211 ever, the most biologically interesting behavior was exhibited by cells achieving a hybrid

212 phenotype, most notable in a dual treatment condition (3C, black arrow), but also present

213 in a small percentage of untreated cells (Fig. 3B, gray arrow). Models with this hybrid

214 phenotype had elevated Sp1 and NFAT transcriptional activity, resulting in simultaneously

215 increased Vimentin and E-cadherin expression (Fig. 4A).

216 To better understand the hybrid phenotype, we simulated the response of the model
217 population to TGF- β 1/2 and VEGF-A treatment with and without NFATc inhibitors (Fig.
218 3). As expected, stimulation with VEGF-A (50 a.u.) maintained an epithelial population
219 (Fig. 3A), while TGF- β 1/2 (10 a.u.) exposure shifted the population from an epithelial
220 to a mesenchymal phenotype (Fig. 3B). On the other hand, combined stimulation with
221 TGF- β 1/2 (10 a.u.) and VEGF-A (50 a.u.) increased both E-cadherin and Vimentin ex-
222 pression, resulting in a hybrid phenotype with both epithelial and mesenchymal character-
223 istics (Fig. 3C). Vimentin expression was correlated with high levels of nuclear phospho-
224 rylated Sp1, following TGF- β 1/2 exposure. Conversely, elevated E-cadherin expression
225 depended upon the activity of NFAT transcription factors downstream of VEGF-A stimula-
226 tion. To further isolate the role of NFAT on this hybrid state, we simulated the inhibition of
227 NFAT transcriptional activity across all conditions (all else being equal). NFAT inhibition
228 in combination with VEGF-A or TGF- β 1/2 treatments blocked increased E-cadherin ex-
229 pression in the case of VEGF-A (Fig. 3D), but did not influence TGF- β 1/2 signaling (Fig.
230 3E). Lastly, NFATc inhibition in combination with simultaneous TGF- β 1/2 and VEGF-A
231 exposure repressed nearly all E-cadherin expression, shifting nearly the entire population
232 towards a mesenchymal phenotype (Fig. 3F). Taken together, high levels of nuclear local-
233 ized phosphorylated Sp1 correlated with Vimentin expression, while NFATc transcriptional
234 activity was critical for maintaining E-cadherin expression in the presence of competing
235 signals.

236 **Combined TGF- β 2 and VEGF-A exposure drives heterogeneity in MCF10A and**
237 **DLD1 cells** The EMT model simulations suggested the transcriptional activity of NFATc
238 and Sp1 could be independently tuned to generate a hybrid cell population with both
239 epithelial and mesenchymal characteristics. To test this hypothesis, we exposed either
240 quiescent epithelial (MCFA10, (Fig. 5)) or transformed epithelial cells (DLD1, (Fig. S2))
241 to combinations of TGF- β 1/2 and/or VEGF-A. As expected, treatment with TGF- β 1/2
242 (10ng/ml) increased Slug and Vimentin expression, while repressing E-cadherin expres-

sion both at the transcript and protein levels in MCF10A (Fig. 5A-B) and DLD1 cells (Fig. S3C, Fig S3 D,E). Both MCF10A (Fig. 5C) and DLD1 cells (Fig. S2E,G) transitioned from quiescent cobblestone morphology to spread spindle shapes, consistent with EMT. As predicted, we found increased nuclear localization of phosphorylated Sp1 following TGF- β 1/2 stimulation in both MCF10A (Fig. 5B,C) and DLD1 cells (Fig. S2E,F). Consistent with model predictions, VEGF-A (50ng/ml) treatment increased the abundance of NFATc1 and E-cadherin at both the transcript and protein level in both MCF10A (Fig. 5A) and DLD1 (Fig. S2A) cells. We also found that NFATc1 nuclear localization significantly increased in both MCF10 and DLD1 treated with VEGF-A independently of the abundance of nuclear localized phosphorylated Sp1 levels (Fig. 5B,C Fig.S3C,E). Interestingly, combining VEGF-A (50ng/ml) with TGF- β 1/2 (10ng/ml) resulted in significantly elevated expression of both E-cadherin and Vimentin at the transcript and protein levels in both MCF10A and DLD1 cells (Fig 5A,B; Fig S3D,E; Fig S4C). NFATc1 expression increased, while Sp1 expression was similar to the TGF- β 1/2 case alone (Fig. 5A-B, Fig S3D,E; Fig S4C)), supporting their independent regulation. The expression of Slug, and Vimentin significantly increased, while E-cadherin levels were increased in MCF10A cells (Fig 5A) and maintained at control levels in DLD1 cells (Fig. S3D). As further predicted, nuclear co-localization of both NFATc1 and phosphorylated Sp1 were apparent in MCF10A and DLD1 cells treated with both ligands (Fig. 5B,C Fig S3E,F). Taken together, combined VEGF-A and TGF- β 1/2 treatment elicited a hybrid phenotype expressing both mesenchymal and epithelial characteristics in both MCF10A and DLD1 cells. This phenotype was driven by the transcriptional activity of two key transcription factors, Sp1 and NFATc, which could be modulated independently by TGF- β 1/2 and VEGF-A exposure.

Our phenotypic analysis predicted that NFATc transcriptional activity was critical to maintaining E-cadherin expression in the presence of both VEGF-A and TGF- β 1/2. We experimentally tested this hypothesis by exposing both MCF10A (Fig. 5E,F) and DLD1 cells (Fig. S3) to combinations of VEGF-A and TGF- β 1/2 in the presence or absence

270 of VIVIT, a soluble peptide inhibitor of NFATc transcriptional activity (Aramburu *et al.*,
271 1999). Treatment with VEGF-A (50ng/ml) and VIVIT (10 μ M) in MCF10A cells significantly
272 reduced E-cadherin expression compared to VEGF-A alone (Fig 5D,E). Co-treatment
273 with VIVIT and TGF- β 1/2 did not enhance EMT capacity of MCF10A cells above that
274 of TGF- β 1/2 alone (Fig 5A,B,E). Likewise, VIVIT in combination with both TGF- β 1/2
275 and VEGF-A resulted in a loss of E-cadherin gene and protein expression, while Slug
276 and Vimentin levels remained increased (Fig. 5D,E). Quantitative flow cytometry con-
277 firmed these results in both MCF10A (Fig. 5F) and DLD1 cells (Fig. S4C). Both epithelial
278 cell lines initially had high levels of E-cadherin expression, and low vimentin abundance
279 (Q1-99.5%), but both MCF10A and DLD1 cells shifted from an epithelial to mesenchymal
280 phenotype (Q1-33.4%, Q4-42.8%) following TGF- β 1/2 exposure. As expected, NFATc
281 nuclear localization was repressed with VIVIT treatment regardless of ligand stimulation,
282 while the abundance of nuclear phosphorylated Sp1 increased for both TGF- β 1/2 and
283 TGF- β 1/2 + VIVIT conditions (Fig. 5D,E). Combined TGF- β 1/2 and VEGF-A increased
284 both Vimentin and E-cadherin expression (Q1-42.1%, Q2-52.3%) compared to TGF- β 1/2
285 alone. Together, these results demonstrate that NFATc and phosphorylated Sp1 are criti-
286 cal for regulating E-cadherin and Vimentin expression during phenotype heterogeneity in
287 MCF10A and DLD1.

288 **Ductal branching during acini formation is dependent upon phenotype heterogene-
289 ity in MCF10A and DLD1 cells** We finally employed established three-dimensional
290 (3D) *in vitro* models of invasion, migration, compaction, and tubulogenesis (Dhimolea
291 *et al.*, 2010) to determine the functional consequences of the hybrid phenotype (Fig. 6).
292 MCF10A and DLD1 cells were aggregated via hanging drop, placed on the surface of a
293 collagen gel, and cultured for 72 hrs under various biochemical treatments. TGF- β 1/2
294 stimulation significantly enhanced cell matrix invasion and matrix compaction, while in
295 contrast VEGF-A stimulation promoted surface migration but no invasion or compaction
296 (Fig. 6B-D). Interestingly, combined TGF- β 1/2 and VEGF-A stimulation significantly in-

297 creased cell migration potential above that of VEGF-A alone while maintaining 3D matrix
298 compaction, though with decreased magnitude compared to TGF- β 1/2 alone. Inhibi-
299 tion of NFATc transcriptional activity by VIVIT decreased migration following treatment
300 with VEGF-A alone (Fig. 6B). Co-treatment of VIVIT significantly decreased migration,
301 while complementarily increasing invasion and compaction, when MCF10A cells were
302 stimulated with both VEGF-A and TGF- β 1/2 (Fig. 6B-D). The responses of DLD1 cells
303 followed a similar trend to MCF10A, although the magnitudes of migration, invasion, and
304 compaction were less. Cell circularity within 3D gels strongly and negatively correlated
305 with both invasion and compaction regardless of treatment (Fig. 6E). Circular-
306 ity index, a common means of quantifying cell morphology, relates cell area to perimeter.
307 A perfect circle has a circularity index equal to 1.0, while a straight line has a circularity
308 index equal to 0.0, see Butcher et al. (Butcher *et al.*, 2004). TGF- β 1/2 treatment alone
309 resulted in irregular and spindle shaped morphology, while VEGF-A exposure promoted
310 round quiescent cells (Fig. 6A). Combined VEGF-A and TGF- β 1/2 promoted morphology
311 between these extremes. VIVIT mediated NFATc inhibition significantly reduced the cir-
312 cularity index, similar to TGF- β 1/2 treatment (Fig. 6F). VEGF-A treatment also induced
313 the formation of tubular structures (acini), but the number of tubular branches relative to
314 total acini was significantly increased upon combined TGF- β 1/2 and VEGF-A. No tubular
315 structures were identified within the DLD1 constructs during the 7 day tubulogenesis end-
316 points, supporting that MCF10A and DLD1 cells have some cell-type specific EMT sensi-
317 tivity despite their underlying competency for acquiring a heterogeneous phenotype. This
318 suggests that initial EMT sensitivity of a cell influences downstream functional response
319 from TGF- β and VEGFA stimulation. Together, these results establish that VEGF-A and
320 TGF- β 1/2 ligand concentrations potentiate between acini and ductal branch formation in
321 3D culture, and are dependent upon NFATc activity.

324 **Discussion**

325 In this study, we developed a family of mechanistic models describing the induction of
326 EMT by TGF- β isoforms in the presence and absence of VEGF-A. The signaling architec-
327 ture encoded in the model, which contained 74 molecular species interconnected by 169
328 interactions, described the expression of 23 genes in response to growth factor stimula-
329 tion. This simulation incorporates an unprecedented level of detail compared to previous
330 models, but as a consequence created a large number of unknown model parameters.
331 Because these parameters could not be estimated uniquely apriori, we estimated an en-
332 semble of likely parameters using the JuPOETs multiobjective optimization framework.
333 The model population was trained and cross-validated to prescribe biological significance
334 using 41 data sets generated in DLD1 colon carcinoma, MDCKII, and A375 melanoma
335 cell lines (Medici *et al.*, 2008). Analysis of this population predicted possible phenotypic
336 modes (and their associated signaling) that cells could exhibit when stimulated with TGF-
337 β and/or VEGF-A. The most novel hypothesis generated from the analysis was that cells
338 could operate in a hybrid state defined by both epithelial and mesenchymal traits when
339 stimulated simultaneously with TGF- β and VEGF-A. We tested this hypothesis in MCF10A
340 and DLD1 cells stimulated with combinations of TGF- β and VEGF-A. As expected, in
341 the presence of TGF- β or VEGF-A alone, MCF10A and DLD1 cells were either mes-
342 enchymal or epithelial, respectively. However, with both TGF- β and VEGF-A, MCF10A
343 and DLD1 cells exhibited a hybrid phenotype, having both epithelial and mesenchymal
344 characteristics. Furthermore, we found that functional traits such as tubulogenesis and
345 ductal branching were different for cells in this hybrid phenotype. Together, this study
346 established a predictive model of EMT induction, determined that deterministic model
347 ensembles could predict population heterogeneity, and proved the existence of a unique
348 hybrid phenotype resulting from the simultaneous integration of extracellular growth factor
349 signals.

350 Cells routinely process a multitude of signals simultaneously, especially when coordi-

nating developmental or pathological programs. For example, oncogenic cells integrate both mechanical and chemical cues in their local microenvironment during tumorigenesis, including cytokines VEGF and TGF- β (Hong *et al.*, 2013). VEGF-A mediates pathological angiogenic remodeling of tumors (Nagy *et al.*, 2007), while TGF- β can elicit both protective and oncogenic responses (Ferrara, 2002, Willis & Borok, 2007). While much research has tested signaling pathways individually, far less is understood about combinatorial stimulation, such as with both VEGF-A and TGF- β . Recent *in vitro* and *in vivo* evidence has suggested that epithelial cells can exhibit heterogeneous phenotypes in addition to classically defined epithelial or mesenchymal states (Polyak & Weinberg, 2009, Strauss *et al.*, 2011). For example, expression profiling in human epithelial cancer cell lines demonstrated a spectrum of phenotypes, including some that expressed both E-cadherin and Vimentin simultaneously (Neve *et al.*, 2006, Welch-Reardon *et al.*, 2014). Zajchowski *et al.*, speculated that these expression profiles were somehow important for maintaining epithelial properties, while simultaneously allowing other functional behavior such as proliferation and migration (Zajchowski *et al.*, 2001). Whether and how heterogeneous phenotypes arise and participate in cancer progression, as well as their response to pharmacological inhibition are fundamental questions that should receive increased attention. In this study, we determined that a hybrid phenotype could be obtained through combined treatment with VEGF-A and TGF- β , both common factors localized in the tumor microenvironment. Furthermore, our systematic simulation-experimentation strategy identified that the transcriptional activity of Sp1 and NFATc were the critical factors controlling this phenotypic heterogeneity. Several studies have highlighted the importance of NFATc as a key transcription factor involved in cell growth, survival, invasion, angiogenesis and cancer (Mancini & Toker, 2009). For example, proliferation and anchorage-independent growth of pancreatic tumor cells is dependent on calcineurin and NFATc1 activity, consistent with the high levels of nuclear NFATc1 found in pancreatic tumors (Singh *et al.*, 2010). Likewise, our results found that VEGF-A was a potent inducer of

378 NFATc1 expression, which may be required for epithelial cell migration and tubulogenesis.
379 Although specific NFATc isoforms were not distinguished in the model, our simulations
380 suggested that NFATc transcriptional activity was capable of maintaining epithelial traits,
381 even during TGF- β induced EMT. Experimentally, we found that E-cadherin expression
382 was dependent upon NFATc dephosphorylation in response to simultaneous VEGF-A and
383 TGF- β 1/2 treatment. Thus, these results support the hypothesis that NFATc activity plays
384 a critical role in maintaining cell-cell contacts, even during partial EMT.

385 Epithelial cells reproduce tissue-like organization when grown in a three-dimensional
386 extracellular matrix (ECM) environment, and therefore are an attractive model to study
387 morphogenic mechanisms. It is well established that MCF10A cells form structures that
388 closely resemble acini (multi-lobed cluster of cells) in three-dimensional *in vitro* cultures
389 (Debnath *et al.*, 2003). It has been postulated that a cellular response reminiscent of
390 partial EMT underlies this process, stimulating further branching and formation of acini
391 (Pearson & Hunter, 2007). Normally well controlled process such as tubulogenesis can
392 be co-opted by cancer cells to break away from a primary lesion and invade through
393 the surrounding stroma (O'Brien *et al.*, 2004). However, by retaining a transient hybrid
394 EMT-like state, clusters of these tube-forming tumor cells can reform at a high rate af-
395 ter invasion, possibly explaining why invasive human carcinomas frequently appear to be
396 cellular collections with varying degrees of gland-like differentiation (Debnath & Brugge,
397 2005). In this study, we showed that our predicted hybrid phenotype generated by sim-
398 taneous treatment of epithelial cells with VEGF-A and TGF- β possessed altered migra-
399 tion and invasion, which enhanced tubular branching. A salient feature of this behavior,
400 however, was the retention of cell-cell contacts that allowed cells to migrate without com-
401 pletely dissociating from their neighbors. Thus, our results support a mechanism in which
402 hybrid cells can maintain some functional characteristics of epithelial cells such as cell-
403 cell adhesion, which are normally lost in a fully differentiated mesenchymal state. The
404 tumor microenvironment contains many soluble signals simultaneously, including VEGF

405 and TGF- β . Thus, it is likely that some cancerous epithelial cells could exhibit hybrid EMT
406 phenotypic states. This may explain why fibroblastoid morphology, a classical feature of
407 EMT, is not commonly observed in human carcinomas (Debnath & Brugge, 2005). This
408 study focused on the combinatorial effects of two very different ligand families present to-
409 gether in the tumor environment. Additional modeling studies are required to unravel the
410 global response of epithelial cells to the full spectrum of chemical, substrate, and mechan-
411 ical cues. The simulation strategy presented here is readily adaptable to larger species
412 sets, with the major advantage that experimentally testable hypotheses can be generated
413 regarding how signals get integrated to produce global cellular response. Furthermore, by
414 simulating multiple ensembles of parameter sets, subpopulations across a constellation of
415 phenotypes can be created and mined for common and/or divergent signaling character-
416 istics. This is a significant advantage over forced convergence to a single unique solution
417 and thereby generating a potentially non-physiological homogeneous population.

418 The deterministic population of EMT models predicted heterogeneous behavior that
419 was qualitatively consistent with experimental studies. There is a diversity of algorithmic
420 approaches to estimate model parameters (Moles *et al.*, 2003), as well as many strategies
421 to integrate model identification with experimental design (Rodriguez-Fernandez *et al.*,
422 2013, Villaverde & Banga, 2014). However, despite these advances, the identification of
423 models describing intracellular network behavior remains challenging. There are differ-
424 ent schools of thought to deal with this challenge. One school has focused on model
425 reduction. Data-driven approaches (Cirit & Haugh, 2012), boolean (Choi *et al.*, 2012) or
426 other logical model formulations (Morris *et al.*, 2011, Terfve *et al.*, 2012) are emerging
427 paradigms that constrain model complexity by the availability of the training and validation
428 data. Other techniques such as constraints based modeling, which is commonly used to
429 model metabolic networks, have also been applied to model transcriptional networks, al-
430 though primarily in lower eukaryotes and prokaryotes (Hyduke & Palsson, 2010). These
431 techniques (and many others, see review (Wayman & Varner, 2013)) are certainly ex-

432 citing, with many interesting properties. Here, we used a traditional approach of mass
433 action kinetics within an ordinary differential equation framework that also included trans-
434 fer functions to simplify scenarios where reactions involving one species are controlled
435 by several others (e.g. E-cadherin transcription). The identification problem for the EMT
436 model was massively underdetermined. This is not uncommon for differential equation
437 models, especially those that are highly mechanistic. Of course, we could have discarded
438 mechanism or reduced the model scope to decrease the complexity of the identification
439 problem. However, a central criticism leveled by biologists is that model simplification is
440 often done at the cost of biological reality, or done for reasons of computational expedi-
441 ency (Sainani, 2012). To avoid this criticism, we systematically identified an ensemble
442 of likely models each consistent with the training data, instead of a single but uncertain
443 best fit model. Previously, we (and others) have suggested that deterministic ensembles
444 could model heterogeneous populations in situations where stochastic computation was
445 not feasible (Lequieu *et al.*, 2011). Population heterogeneity using deterministic model
446 families has previously been explored for bacterial growth in batch cultures (Lee *et al.*,
447 2009). In that case, distributions were generated because the model parameters var-
448 ied over the ensemble, i.e., extrinsic noise led to population heterogeneity. In this study,
449 parameters controlling physical interactions such as disassociation rates or the rate of
450 assembly or degradation of macromolecular machinery such as ribosomes were widely
451 distributed over the ensemble. Population heterogeneity can also arise from intrinsic ther-
452 mal fluctuations, which are not captured by a deterministic population of models (Swain
453 *et al.*, 2002). Thus, deterministic ensembles, provide a coarse-grained or extrinsic-only
454 ability to simulate population diversity. Despite this limitation, our prediction of phenotypic
455 heterogeneity (and the underlying signaling events responsible for the heterogeneity) was
456 consistent with experimental observations. This suggested that deterministic ensembles
457 could simulate disease or developmental processes in which heterogeneity plays an im-
458 portant role, without having to resort to stochastic simulation.

A common criticism of ODE modeling has been the poorly characterized effect of structural and parametric uncertainty. In this study, parametric uncertainty was addressed by developing an ensemble of probable models instead of a single best-fit but uncertain model using multiobjective optimization. While computationally complex, multiobjective optimization is an important tool to address qualitative conflicts in training data that arise from experimental error or cell line artifacts (Handl *et al.*, 2007). On the other hand, structural uncertainty is defined as uncertainty in the biological connectivity. The EMT model connectivity was assembled from an extensive literature review. However, several potentially important signaling mechanisms were not included. First, we identified a potential gap in biological knowledge surrounding the regulation of LEF-1 expression, that was filled by the addition of the hypothetical YREG1 transcriptional repressor. The LEF-1 transcription factor is expressed in tissues that undergo EMT during embryogenesis (Nawshad & Hay, 2003, Vega *et al.*, 2004), and has been suggested to promote an invasive phenotype in cancer cells (Cano *et al.*, 2000, Kim *et al.*, 2002). Low levels of YREG1 were important for stabilizing the interaction between LEF-1 and β -catenin, while elevated levels inhibited EMT by downregulating LEF-1 transcriptional activity. Recent evidence has established a complex role of Amino terminal Enhancer of Split (AES) and Groucho/TLE on suppressing LEF-1 activity. AES opposes LEF-1 transcriptional activation while Groucho/TLE binds with LEF-1 for a histone deacetylase repression. In addition, β -catenin directly displaces Groucho/TLE repressors from TCF/LEF-1 in Wnt-mediated transcription activation (Arce *et al.*, 2009, Grumolato *et al.*, 2013). Our model agrees with this newly discovered feedback system, as YREG1 regulates LEF-1 activity leading to EMT stabilization.

Recent evidence has also suggested an essential role of NF- κ B in epithelial transformation. NF- κ B may influence Snail expression through the AKT pathway and directly stabilize Snail activity (Wu *et al.*, 2009). This is particularly important for integrating inflammation pathways, such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), which have been linked to EMT in pathological conditions (Sullivan *et al.*, 2009). Other

486 pathways such as Notch have also been shown to act synergistically with TGF- β to ex-
487 press Slug in the developing embryo (Niessen *et al.*, 2008). Lastly, while we have modeled
488 classical protein signaling, we have not considered the role of regulatory RNAs on EMT.
489 There is growing evidence that microRNAs (miRNAs) play a strong role in EMT, where
490 several miRNAs, for example miR-21 and miR-31 are strongly associated with TGF- β ex-
491 posure (Bullock *et al.*, 2012). Addressing missing structural components like these, could
492 generate more insight into TGF- β signaling and its role in phenotypic transformation.

493 **Materials and Methods**

494 The model code and parameter ensemble is freely available under an MIT software li-
495 cense and can be downloaded from <http://www.varnerlab.org>.

496 **Signaling network connectivity** The EMT model described the gene expression pro-
497 gram resulting from TGF- β and VEGF-A signaling in a prototypical epithelial cell. The
498 TGF- β -EMT network contained 97 nodes (proteins, mRNA or genes) interconnected by
499 251 interactions. The network connectivity was curated from more than 40 primary liter-
500 ature sources in combination with on-line databases (Jensen *et al.*, 2009, Linding *et al.*,
501 2007). The model interactome was not specific to a single epithelial cell line. Rather, we
502 assembled canonical pathways involved in TGF- β and VEGF-A signaling, defaulting to
503 human connectivity when possible. Using a canonical architecture allowed us to explore
504 general features of TGF- β induced EMT without cell line specific artifacts. On the other
505 hand, because of the canonical architecture, we can test the model against several cell
506 lines to test the generality of our conclusions.

507 Our signaling network reconstruction was based on Medici *et al.* who identified the
508 pathways through which MDCKII, DLD1 colon carcinoma, and A375 melanoma cells tran-
509 sition towards a mesenchymal phenotype (Medici *et al.*, 2008). Sequential activation of
510 MAPK and Smad pathways were initiated upon addition of TGF- β 1/2. Briefly, TGF- β 2
511 signals through the RAS-RAF-MEK-ERK pathway to up-regulate Snail and Slug expres-
512 sion (Medici *et al.*, 2006). Snail, a known repressor of junctional proteins, inhibits the ex-
513 pression of E-cadherin (Cano *et al.*, 2000). This initial repression of E-cadherin leads to a
514 release of β -catenin from the cell membrane. This release of β -catenin can then translo-
515 cate to the nucleus and form transcriptional complexes with TCF-4 to drive TGF- β 3 ex-
516 pression (Medici *et al.*, 2008). The PI3K to GSK3 pathway was included and acted as
517 an activating mechanism of β -catenin signaling through TGF- β 3 signaling (Medici *et al.*,
518 2008). GSK3 is known to act on β -catenin signaling through the ubiquitin-proteasome
519 pathway (Larue & Bellacosa, 2005, Zhou *et al.*, 2004). Thereby, further β -catenin release

520 also resulted from by TGF- β 3 signals to the cells interior by binding to type II receptors,
521 which form heterodimers with type I receptors (ALK5) (Derynck & Zhang, 2003). This
522 activates the receptors serine/threonine kinase activity to phosphorylate and activate the
523 receptor Smads 2/3 (Massagué *et al.*, 2005). In the model, receptors are simplified and
524 represented as either bound or unbound complexes with their ligands. Phosphorylated
525 Smads 2/3 (pSmad2/3) form heterodimers and translocate to the nucleus. pSmads com-
526 plexes up-regulate other transcription factors, such as LEF-1. The pSmad2/4-LEF-1 com-
527 plex has been shown to directly repress the E-cadherin gene (Nawshad *et al.*, 2007).
528 LEF-1 also binds with β -catenin to upregulate mesenchymal proteins such as fibronectin
529 (Medici *et al.*, 2011). In the model, Smad signaling is consolidated into a single Smad
530 species that can act in a co-dependent fashion with LEF1 to downregulate E-cadherin via
531 a transfer function, eliminating the need for an explicitly LEF-1, pSmad complex. The EMT
532 gene expression program was initiated by the binding of TGF- β isoforms to TGF- β sur-
533 face receptors, starting the downstream signaling program. Repression of E-cadherin
534 expression is the central event in the transition from an epithelial to a mesenchymal
535 phenotype (Cano *et al.*, 2000). However, this transition is not solely driven by transcrip-
536 tional events. At the protein level, the repression of E-cadherin leads to a release of
537 β -catenin from cell membrane. Cytosolic β -catenin then translocates to the nucleus and
538 forms transcriptionally-active complexes with immunoglobulin transcription factor 2 (TCF-
539 4) to drive TGF- β 3 expression (Medici *et al.*, 2008). The PI3K to GSK3 pathway was
540 included and acted as an activating mechanism of β -catenin signaling through TGF- β 3
541 signaling (Medici *et al.*, 2008). GSK3 is known to act on β -catenin signaling through APC
542 complex associated ubiquitin-proteasome pathway. The APC complex is represented in
543 our model and serves as a second reservoir of β -catenin in untransformed cells whose
544 sequestration is regulated by GSK3 (Larue & Bellacosa, 2005, Medici *et al.*, 2008, Zhou
545 *et al.*, 2004). Lastly, VEGF-A activation of NFATc1 takes place through calcineurin sig-
546 naling leading to an enhancement of E-cadherin expression (Suehiro *et al.*, 2014), as

547 supported by our VEGF-A experimental data (Fig. S4).

548 **Formulation, solution and analysis of the EMT model equations**

549 *EMT signaling events.* EMT signaling events were modeled using either saturation or
550 mass-action kinetics within an ordinary differential equation (ODE) framework:

$$\frac{1}{\tau_i} \frac{dx_i}{dt} = \sum_{j=1}^{\mathcal{R}} \sigma_{ij} r_j(\mathbf{x}, \epsilon, \mathbf{k}) - \mu x_i \quad i = 1, 2, \dots, \mathcal{M} \quad (1)$$

551 where \mathcal{R} denotes the number of signaling reactions and \mathcal{M} denotes the number of pro-
552 teins in the model. The quantity τ_i denotes a time scale parameter for species i which
553 captures unmodeled effects. In the current study $\tau_i = 1$ for all species. The quantity
554 $r_j(\mathbf{x}, \epsilon, \mathbf{k})$ denotes the rate of reaction j . Typically, reaction j is a non-linear function of
555 biochemical and enzyme species abundance, as well as unknown model parameters \mathbf{k}
556 ($\mathcal{K} \times 1$). The quantity σ_{ij} denotes the stoichiometric coefficient for species i in reaction j . If
557 $\sigma_{ij} > 0$, species i is produced by reaction j . Conversely, if $\sigma_{ij} < 0$, species i is consumed
558 by reaction j , while $\sigma_{ij} = 0$ indicates species i is not connected with reaction j . Species
559 balances were subject to the initial conditions $\mathbf{x}(t_o) = \mathbf{x}_o$.

560 Rate processes were written as the product of a kinetic term (\bar{r}_j) and a control term (v_j)
561 in the EMT model. The rate of enzyme catalyzed reactions was modeled using saturation
562 kinetics:

$$\bar{r}_j = k_j^{cat} \epsilon_i \left(\frac{x_s}{K_{js} + x_s} \right) \quad (2)$$

563 where k_j^{cat} denotes the catalytic rate constant for reaction j , ϵ_i denotes the abundance of
564 the enzyme catalyzing reaction j , and K_{js} denotes the saturation constant for species s
565 in reaction j . On the other hand, mass action kinetics were used to model protein-protein
566 binding interactions within the network:

$$\bar{r}_j = k_j^{max} \prod_{s \in m_j^-} x_s^{-\sigma_{sj}} \quad (3)$$

567 where k_j^{max} denotes the maximum rate for reaction j , σ_{sj} denotes the stoichiometric coefficient for species s in reaction j , and $s \in m_j$ denotes the set of *reactants* for reaction j .
 568 We assumed all binding interactions were irreversible.

570 The control terms $0 \leq v_j \leq 1$ depended upon the combination of factors which influenced rate process j . For each rate, we used a rule-based approach to select from competing control factors. If rate j was influenced by $1, \dots, m$ factors, we modeled this relationship as $v_j = \mathcal{I}_j(f_{1j}(\cdot), \dots, f_{mj}(\cdot))$ where $0 \leq f_{ij}(\cdot) \leq 1$ denotes a regulatory transfer function quantifying the influence of factor i on rate j . The function $\mathcal{I}_j(\cdot)$ is an integration rule which maps the output of regulatory transfer functions into a control variable. In this study, we used $\mathcal{I}_j \in \{\min, \max\}$ (Sagar & Varner, 2015). If a process has no modifying factors, $v_j = 1$.

578 *EMT gene expression processes.* The EMT model described both signal transduction
 579 and gene expression events following the addition of TGF- β and VEGF-A. For each
 580 gene, we modeled both the resulting mRNA (m_j) and protein (p_j):

$$\frac{dm_j}{dt} = r_{T,j} - (\mu + \delta_{m,j}) m_j + \lambda_j \quad (4)$$

$$\frac{dp_j}{dt} = r_{X,j} - (\mu + \delta_{p,j}) p_j \quad (5)$$

581 where $j = 1, 2, \dots, \mathcal{G}$. The terms $r_{T,j}$ and $r_{X,j}$ denote the specific rate of transcription,
 582 and translation while the terms $\delta_{m,j}$ and $\delta_{p,j}$ denote degradation constants for mRNA and
 583 protein, respectively. Lastly, μ denotes the specific growth rate, and λ_j denotes the con-
 584 stitutive rate of gene expression for gene j . The specific transcription rate was modeled
 585 as the product of a kinetic term $\bar{r}_{T,j}$ and a control term u_j which described how the abun-
 586 dance of transcription factors, or other regulators influenced the expression of gene j .
 587 The kinetic rate of transcription was modeled as:

$$\bar{r}_{T,j} = \alpha_j \left[V_T^{max} \left(\frac{G_j}{K_T + G_j} \right) \right] \quad (6)$$

588 where the maximum gene expression rate was defined as the product of a character-
 589 istic transcription rate constant (k_T) and the abundance of RNA polymerase, $V_T^{max} =$
 590 $k_T (RNAP)$. The parameter α_j was used to adjust the transcription to that of gene j (es-
 591 timated in this study), while k_T , G_j and $RNAP$ were estimated from literature (Milo *et al.*,
 592 2010). Similar to the signaling processes, the gene expression control term $0 \leq u_j \leq 1$
 593 depended upon the combination of factors which influenced rate process j . For each
 594 rate, we used a rule-based approach to select from competing control factors. If the ex-
 595 pression of gene j was influenced by $1, \dots, m$ factors, we modeled this relationship as
 596 $u_j = \mathcal{I}_j(f_{1j}(\cdot), \dots, f_{mj}(\cdot))$ where $0 \leq f_{ij}(\cdot) \leq 1$ denotes a regulatory transfer function
 597 quantifying the influence of factor i on the expression of gene j . The function $\mathcal{I}_j(\cdot)$ is an
 598 integration rule which maps the output of regulatory transfer functions into a control vari-
 599 able. In this study, we used $\mathcal{I}_j \in \{\min, \max\}$ (Sagar & Varner, 2015). If a gene expression
 600 process has no modifying factors, $u_j = 1$. Lastly, the specific translation rate was modeled
 601 as:

$$r_{X,j} = \beta_j \left[V_X^{max} \left(\frac{m_j}{K_X + m_j} \right) \right] \quad (7)$$

602 where V_X^{max} denotes a characteristic maximum translation rate estimated from literature,
 603 β_j denotes the transcript specific correction the characteristic translation rate, and K_X
 604 denotes a translation saturation constant. The characteristic maximum translation rate
 605 was defined as the product of a characteristic translation rate constant (k_X) and the abun-
 606 dance of Ribosomes ($RIBO$), $V_X^{max} = k_X (RIBO)$, where both k_X and $RIBO$ abundance
 607 were estimated from literature (Milo *et al.*, 2010).

608 The signaling and gene expression model equations were implemented in Julia and
 609 solved using the CVODE routine of the Sundials package (Bezanson *et al.*, 2014, Hindmarsh
 610 *et al.*, 2005). The model code and parameter ensemble is freely available under an MIT
 611 software license and can be downloaded from <http://www.varnerlab.org>.

612 *Estimation of model parameters using multiobjective optimization.* The EMT model had
 613 296 unknown parameters (169 kinetic constants, 44 saturation constants, 38 control logic

paramters, and 45 non-zero initial conditions) which were not uniquely identifiable given the training data. Instead, we estimated a population of likely models (each consistent with the training data) using 41 data sets generated in DLD1 colon carcinoma, MDCKII, and A375 melanoma cells taken from Medici *et al.* (Medici *et al.*, 2008). We used the Pareto Optimal Ensemble Technique (JuPOETs) multiobjective optimization framework in combination with leave-one-out cross-validation to estimate an ensemble of model parameters (Song *et al.*, 2010). Cross-validation was used to calculate both training and prediction error during the parameter estimation procedure (Kohavi, 1995). The 41 intracellular protein and mRNA data-sets used for identification were organized into 11 objective functions. These 11 objective functions were then partitioned, where each partition contained ten training objectives and one validation objective. The training and validation data were Western blots. We achived a physical simulation scale by establishing characteristic rates of transcription, translation, mRNA and protein degradation, as well as characteristic concentrations of ribosomes and RNAPs (Milo *et al.*, 2010). The concentration scale is in nM, with proteins ranging from 10-1000nM and mRNA ranging from 0.01 to 1nM, reflecting the true abundances and ratios between each species.

Cell culture and experimental interrogation DLD1 colon carcinoma, MCF10A, and HUVEC were acquired from the American Tissue Culture Collection (Manassas, VA). Cells were grown in culture with RPMI 1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin for DLD1, EBM-2 supplemented with EGM-2, 5% fetal bovine serum, and 1% penicillin/streptomycin for HUVEC, or MGEM 2 supplemented with insulin, bovine pituitary extract, cholera toxin, hEGF, hydrocortisone, 5% horse serum, and 1% penicillin/streptomycin for MCF10A. Cells were serum starved for 24 hours and removed from all experimental conditions. Recombinant VEGFA165 was also removed from culture medium prior to experimentation. Recombinant human TGF- β 2 (R & D Systems, Minneapolis, MN) was added to the culture medium at a concentration of 10 ng/ml and recombinant VEGFA165 at a concentration of (5ng/ml, 50ng/ml) for all relative experiments.

641 NFAT inhibitor (VIVIT peptide) (EMDBiosciences, Darmstadt, Germany), was added to
642 the culture medium at a concentration of 10 μ 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 *VEGF treatment* DLD1 and MCF10A cells were suspended in culture media (with RPMI
645 1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin for DLD1 or
646 MGEM 2 supplemented with insulin, bovine pituitary extract, cholera toxin, hEGF, hydro-
647 cortisone, 5% horse serum, and 1% penicillin/streptomycin for MCF10A), and allowed to
648 aggregate overnight in hanging drop culture (20 μ L; 20,000 cells). The spherical aggre-
649 gates were placed on the surface of neutralized type I collagen hydrogels (1.5mg/mL)
650 and allowed to adhere. Cultures were then serum starved (1% serum) for 24 hours. Re-
651 combinant VEGFA165 was then added to the media (5ng/ml, 50ng/ml) and mRNA was
652 harvested after 3hr and 24hr timepoint.

653 *RT-PCR* RNA extractions were performed using a Qiagen total RNA purification kit (Qi-
654 agen, Valencia, CA) and RNA was reverse transcribed to cDNA using the SuperScript
655 III RT-PCR kit with oligo(dT) primer (Invitrogen). Sufficient quality RNA was determined
656 by an absorbance ratio A260/A280 of 1.8-2.1, while the quantity of RNA was determined
657 by measuring the absorbance at 260nm (A260). Real-time PCR experiments were con-
658 ducted using the SYBR Green PCR system (Biorad, Hercules, CA) on a Biorad CFX96
659 cycler, with 40 cycles per sample. Cycling temperatures were as follows: denaturing,
660 95C; annealing, 60C; and extension, 70C. Primers were designed to detect GAPDH, E-
661 cadherin, vimentin, Slug, Sp1, and NFATc1 in cDNA clones: Sp1 (F-TTG AAA AAG GAG
662 TTG GTG GC, R-TGC TGG TTC TGT AAG TTG GG, Accession NG030361.1), NFATc1
663 (F-GCA TCA CAG GGA AGA CCG TGT C, R-GAA GTT CAA TGT CGG AGT TTC TGA
664 G, Accession NG029226.1). GAPDH, E-cadherin, vimentin, and Slug primers were taken
665 from previously published literature (Medici *et al.*, 2008).

666 *Antibody Staining* Samples were fixed in 4% PFA overnight at 4C. Samples were then
667 washed for 15 minutes on a rocker 3 times with PBS, permeabilized with 0.2% Triton-X

668 100 (VWR International, Radnor, PA) for 10 minutes, and washed another 3 times with
669 PBS. Samples were incubated overnight at 4C in a 1% BSA (Rockland Immunochemi-
670 cals, Inc., Gilbertsville, PA) blocking solution followed by another 4C overnight incubation
671 with either rabbit anti-human E-cadherin 1:100 (Abcam, ab53033), mouse anti-human
672 phospho-Sp1 1:100 (Abcam, ab37707), mouse anti-human vimentin 1:100 (Invitrogen,
673 V9), and rabbit anti-human NFATc1 (Santa Cruz, sc-7294) 1:100. After 3 washes for 15
674 minutes with PBS, samples were exposed to Alexa Fluor 488 or 568 conjugated (Invit-
675 rogen), species specific secondary antibodies at 1:100 in 1% BSA for 2 hours at room
676 temperature. Three more washes with PBS for 15 minutes were followed by incubation
677 with either DRAQ5 far red nuclear stain (Enzo Life Sciences, Plymouth Meeting, PA) at
678 1:1000.

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

689 *Three-Dimensional Culture and Tubulogenesis Assays* For invasion/migration assays,
690 cells were resuspended in culture media, and allowed to aggregate overnight in hanging
691 drop culture (20 μ L; 20,000 cells). The spherical aggregates were placed on the surface of
692 neutralized type I collagen hydrogels (1.5mg/mL) and allowed to adhere for 2 hrs before
693 adding treatments. Cultures were maintained for 72 hrs, after which they were fixed in
694 4% PFA and slowly rehydrated using PBS. For compaction assays, cells were pelleted

695 via centrifugation and resuspended within a neutralized collagen hydrogel (1.5mg/mL)
696 solution at a density of 400,000 cells/mL. 250 μ L of gel was inoculated into culture wells,
697 which solidified after 60min. Treatments were then added within 800 μ L of the culture
698 medium without serum. Gels were liberated from the surfaces of the culture wells the
699 next day and cultured free floating for an additional 3-7 days, exchanging serum free
700 media with appropriate factors every 48 hrs.

701 Tubulogenesis was defined as a typical nonmalignant acini structure. This includes a
702 polarized epithelial cell, hollow lumen, and the basal sides of the cell are surrounded by
703 ECM proteins (Fig. 6A, Controls or VEGF treated). Previous work has shown that change
704 in the morphological characteristics of nontumorigenic MCF10A epithelial acini occur over
705 time and exploiting them to growth in 3D culture can be quantified. For example, using
706 image segmentation, Chang et al. (Chang *et al.*, 2007) examined the elongation of the
707 MCF10A acini at 6, 12, and 96 hours after a particular treatment. Polizzotti et al. (Poliz-
708 zotti *et al.*, 2012) also suggested a computational method to quantify acini structure based
709 on morphological characteristics in nonmalignant, noninvasive, and invasive conditions.
710 Adapted from these approaches, we first fluorescently labeled our cultures and captured
711 the acini structures by 3D confocal microscopy. Next individual acini structures in the im-
712 ages were segmented by imageJ and labeled. We then extracted the number of ductal
713 branches. Ductal branching was defined as any elongated cell cluster extending away
714 from the total acini structure, which was manually segmented and counted using ImageJ.
715 A total of 5 images for each condition were used, and approximately 12 acini were ana-
716 lyzed in each image. Total branching was normalized to the amount of acini present, and
717 provides an overall general assessment to the extent of acini remodeling.

718 *Statistics* Results are expressed as mean \pm standard error, $n \geq 6$. Data was analyzed
719 with the GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego,
720 CA) and SAS (Statistical Analysis Software, Cary, NC). A one-way ANOVA with Tukey's
721 post hoc was used to compare differences between means and data was transformed

⁷²² when necessary to obtain equal sample variances. Differences between means were
⁷²³ considered significant at p<0.05.

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Extracellular

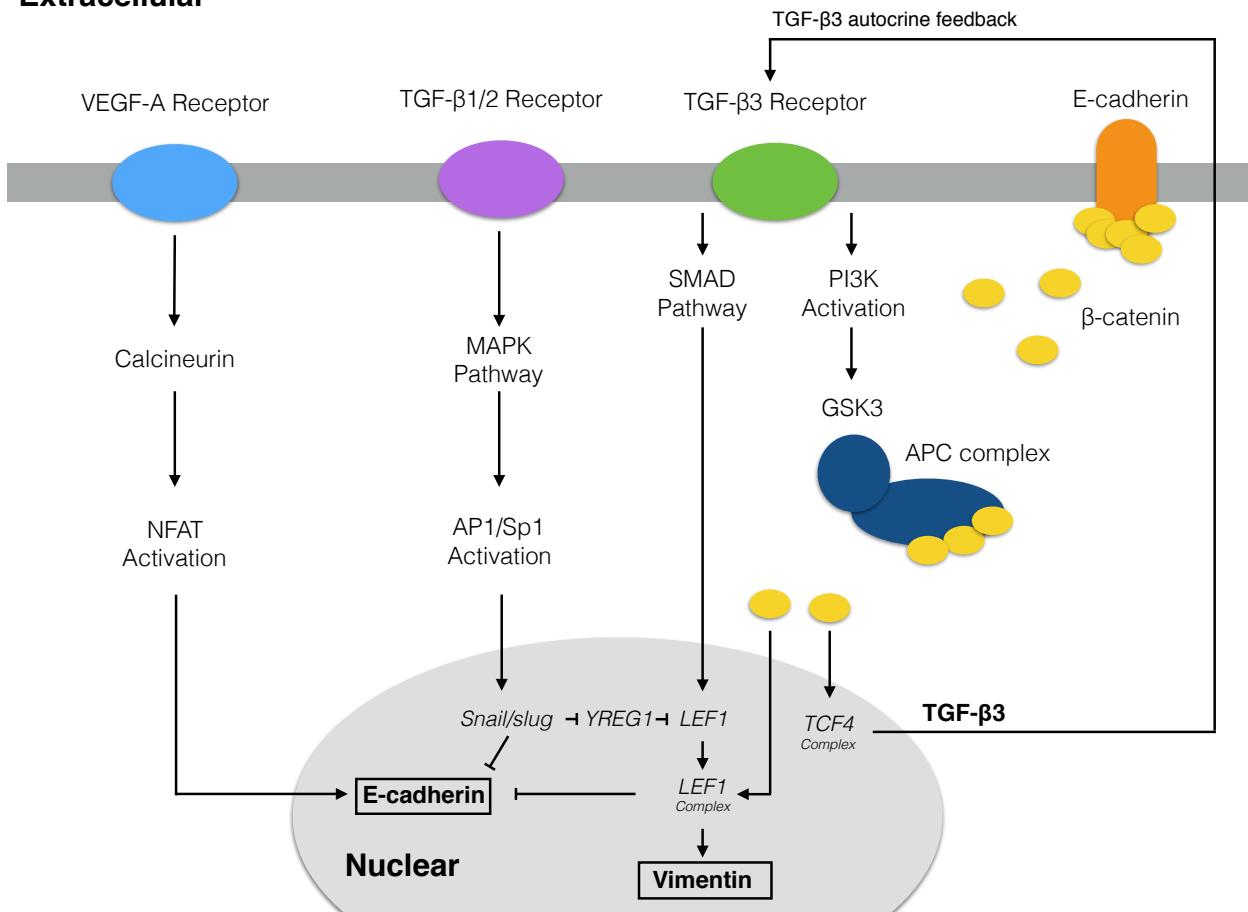


Fig. 1: Model connectivity recreates the core architecture during EMT. The EMT network contains 97 nodes (proteins, mRNA, and genes) interconnected by 169 interactions. Central to EMT induction, activation of the MAPK cascade occurs through TGF- β 1/2 binding which activates the AP-1/Sp1 transcriptional axis. AP-1/Sp1 drives an autocrine response of TGF- β 3, which activates the Smad cascade, leading to phenotypic change. Conversely, VEGF-A binding can stabilize an epithelial phenotype through NFAT activation. Downstream activation of β -catenin signaling due to E-cadherin loss and GSK3 inactivation of β -cateinin confinement is critical to the complete activation of the EMT program. The complete list of molecular interactions that comprise the model is given in the supplement.

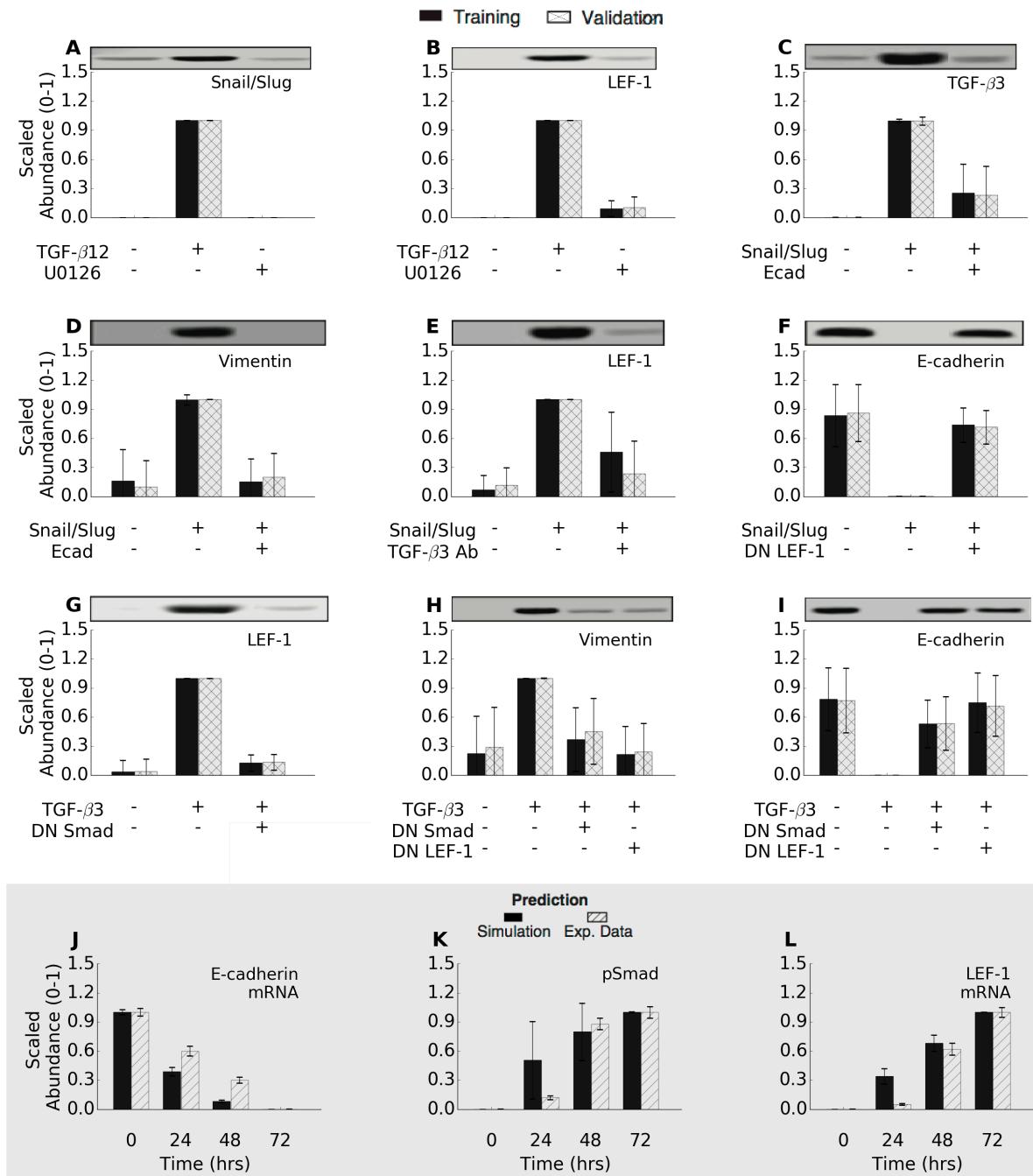


Fig. 2: Training and validation simulations. The population of EMT models qualitatively captured TGF- β -induced EMT signaling. (A-I) The population was generated using JuPOETs and trained using 11 different objective functions (41 data sets) taken from Medici *et al.* (Medici *et al.*, 2008). The model captured the simulated experiments for all cases when compared to randomized controls. (J-L) The model populations were also compared against untrained temporal data to measure the effectiveness as a pure prediction.

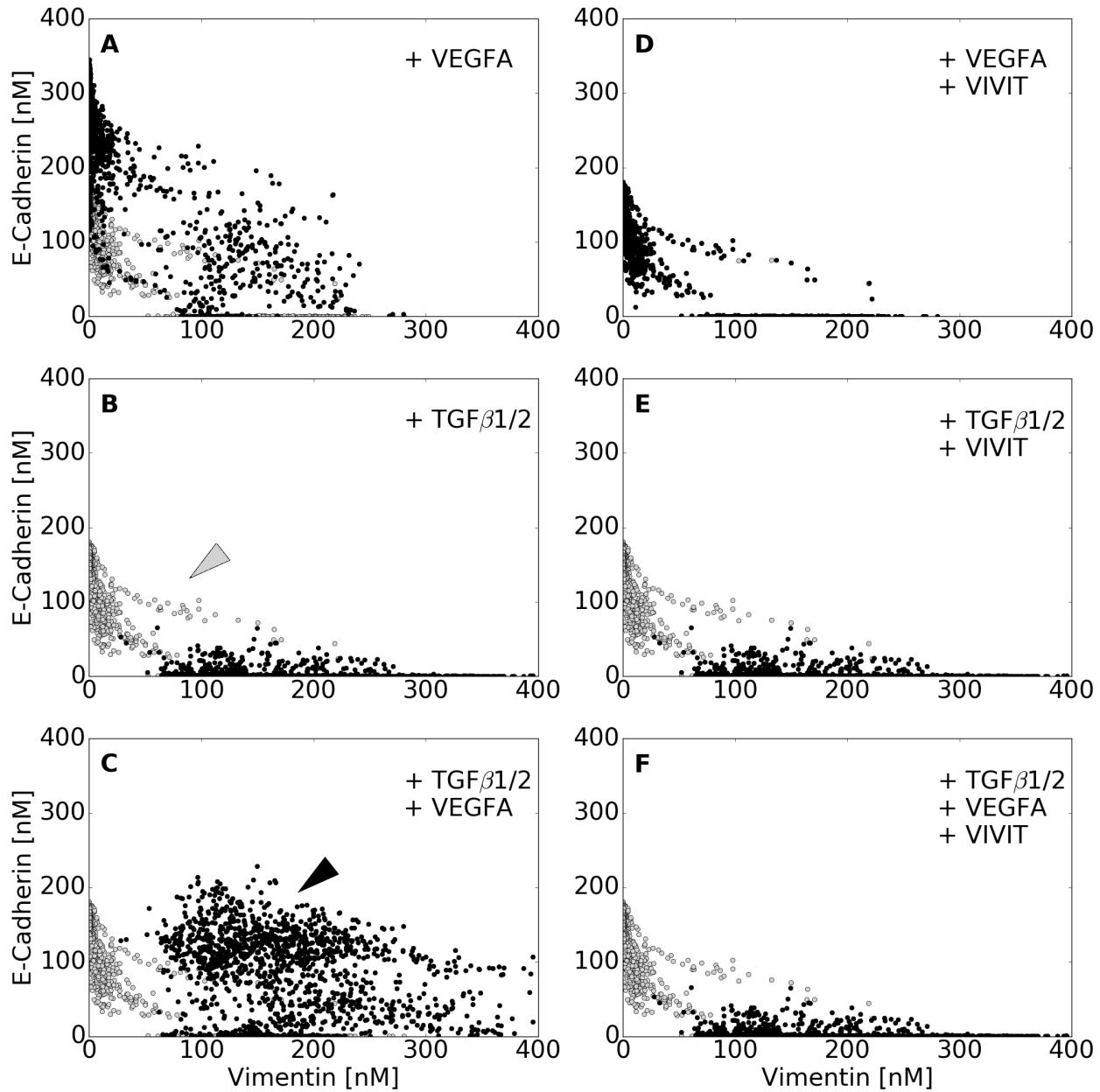


Fig. 3: Simulated VEGF-A and TGF- β 1/2 exposure promoted phenotype heterogeneity. Simulated response to TGF- β 1/2 and VEGF-A exposure with and without axis specific inhibitors. Vimentin and E-cadherin abundances (in nM) were used to quantify the shift in population at 48 hrs. (A-C) VEGF-A (50 a.u.) treatment resulted in a population with enhanced epithelial properties. This was contrary to the addition of TGF- β 2 (10 a.u.), which shifted the population towards a mesenchymal phenotype. Interestingly, the combined effects of TGF- β 2 and VEGFA was found to increase both ecadherin and vimentin levels, creating a heterogeneous population (black arrow), which can also be seen in a minority of untreated cells (gray arrow). (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. Likewise, combining NFAT inhibition with TGF- β mitigated all VEGF enhanced ecadherin expression. Lastly, combination of TGF- β 2, VEGFA, and NFAT inhibition nearly mitigated all effects of VEGFA, shifting the heterogeneous population towards a mesenchymal phenotype. In whole, high levels of phosphorylated-Sp1 correlated with vimentin expression, while NFAT was responsible for maintaining E-cadherin expression in the presence of other factors, although neither were mutually exclusive.

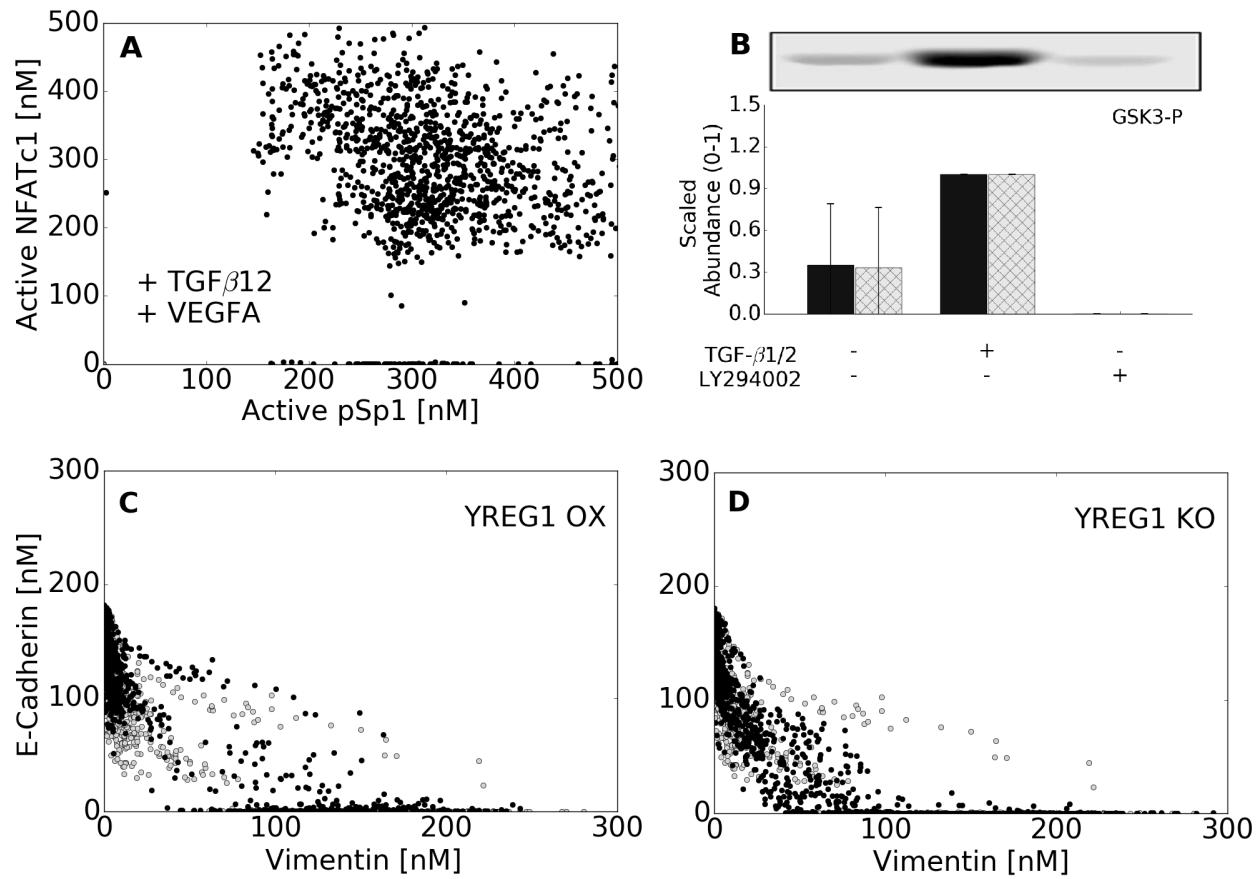


Fig. 4: Analysis of underlying signaling responses. (A) We examined the distribution of NFATc1 and AP1/SP1 in cells containing the hybrid phenotype (VEGF-A + TGF- β 2 case), showing the potential for cells to express both SP1 and NFATc1 in a non exclusive manner. (B) We were able to show a fit to an additional objective demonstrating the activation of GSK3 through PI3K. Our model captures this activation through TGF- β 3 signaling. LY294002 is a PI3K inhibitor. (C) We identified a novel regulator of LEF1 called YREG1 that allows Snail/Slug to emulate an inducer by repressing YREG1, which was required to stabilize the untreated population. YREG1 overexpression revealed an enhanced epithelial phenotype, while some inherently transformed cells moved towards a hybrid phenotype. (D) In the absence of YREG1, most of the population failed to consistently retain a stable epithelial state.

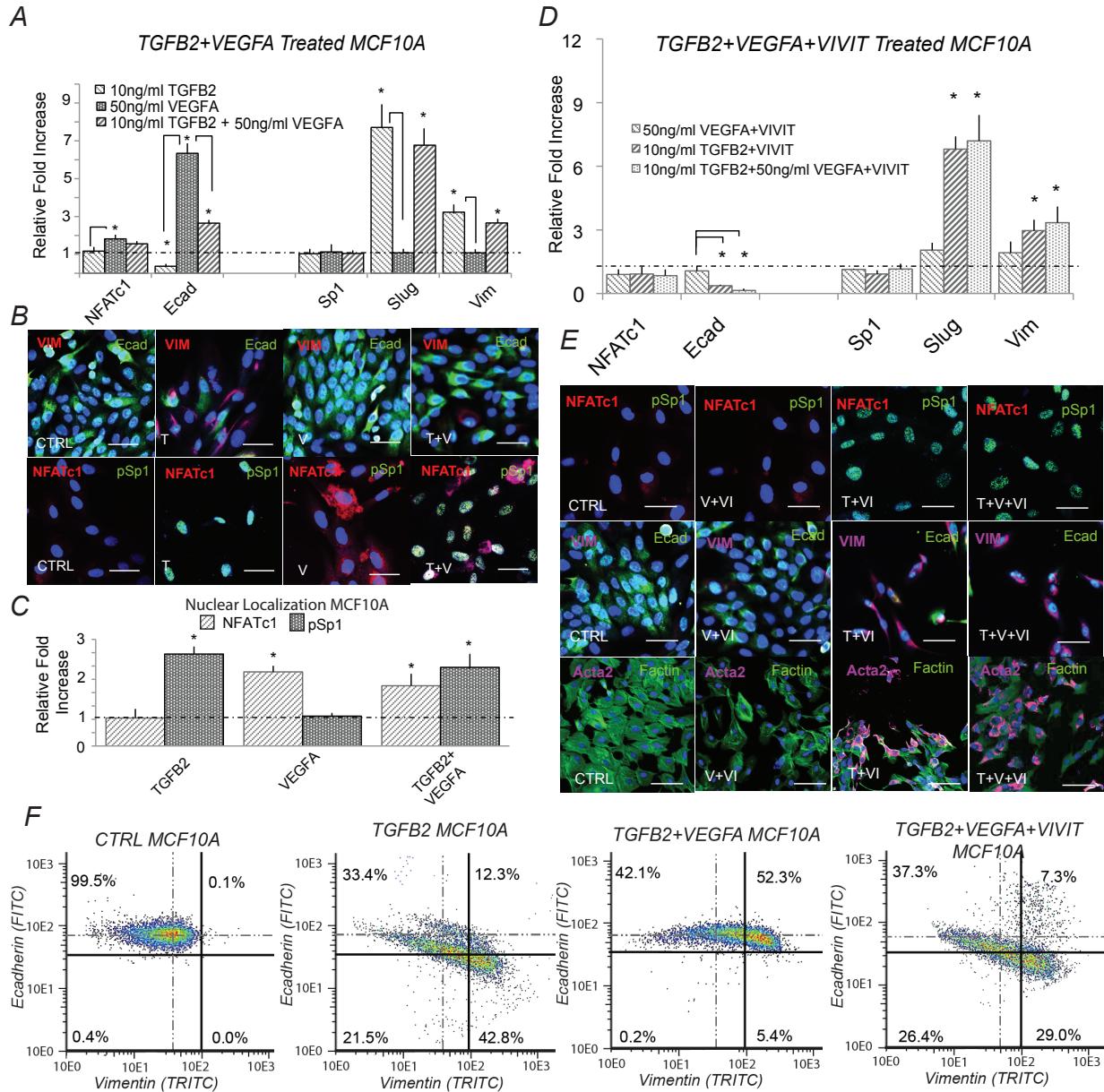


Fig. 5: Simultaneous TGF- β 1/2 and VEGF-A treatment induced phenotype heterogeneity and is dependent upon NFAT activity *in-vitro*. (A) In MCF10A, treatment with (10ng/ml) TGF- β 2 increased Slug and vimentin, while ecadherin expression was inhibited at both the gene and protein level at 48 hrs. Conversely,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

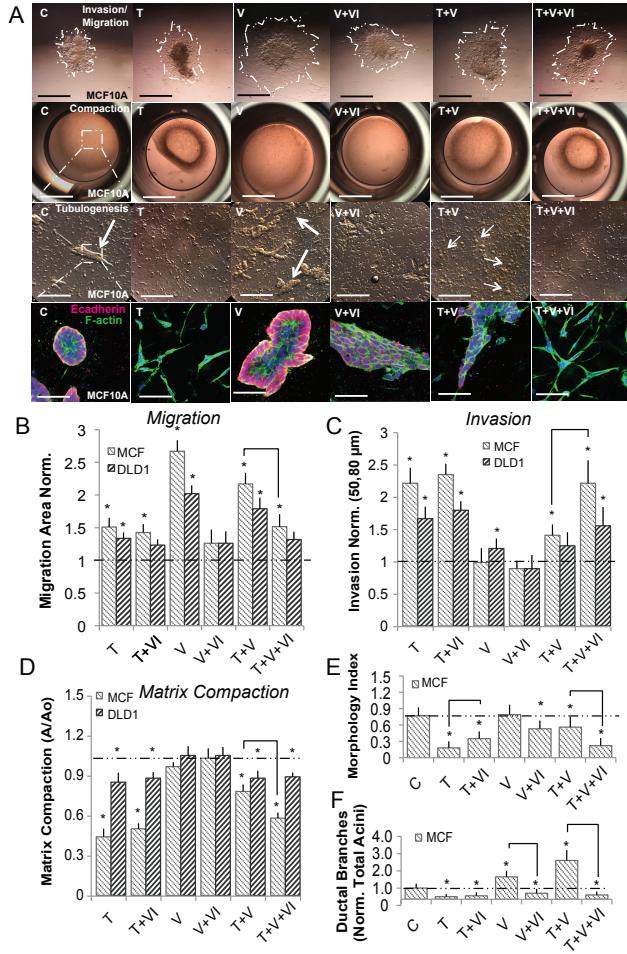


Fig. 6: Ductal branching is dependent upon phenotype heterogeneity within MCF10A in 3-D culture. MCF10A and DLD1 were formed into spheroids overnight and explanted to a collagen gel for 72 hrs. For compaction and tubular assays, cells were embedded into collagen gels for 72 hrs, and the extent of tubulogenesis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

968 **Supplemental Materials and Methods**

969 **Estimation and cross-validation of EMT model parameters.** We used the Pareto Opt-
970 imal Ensemble Technique (POETs) multiobjective optimization framework in combina-
971 tion with leave-one-out cross-validation to estimate an ensemble of TGF- β /EMT models.
972 Cross-validation was used to calculate both training and prediction error during the pa-
973 rameter estimation procedure (Kohavi, 1995). The 41 intracellular protein and mRNA
974 data-sets used for identification were organized into 11 objective functions. These 11
975 objective functions were then partitioned, where each partition contained ten training ob-
976 jectives and one validation objective. POETs integrates standard search strategies e.g.,
977 Simulated Annealing (SA) or Pattern Search (PS) with a Pareto-rank fitness assignment
978 (Bassen *et al.*, 2016, Song *et al.*, 2010). Denote a candidate parameter set at iteration
979 $i + 1$ as \mathbf{k}_{i+1} . The squared error for \mathbf{k}_{i+1} for training set j was defined as:

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

980 The symbol $\hat{\mathcal{M}}_{ij}$ denotes scaled experimental observations (from training set j) while
981 \hat{y}_{ij} denotes the scaled simulation output (from training set j). The quantity i denotes
982 the sampled time-index and \mathcal{T}_j denotes the number of time points for experiment j . In
983 this study, the experimental data used for model training was typically the band intensity
984 from Western or Northern blots. Band intensity was estimated using the ImageJ software
985 package Abramoff *et al.* (2004). The scaled measurement for species x at time $i =$
986 $\{t_1, t_2, \dots, t_n\}$ in condition j is given by:

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

987 Under this scaling, the lowest intensity band equaled zero while the highest intensity
988 band equaled one. A similar scaling was defined for the simulation output. By doing this
989 scaling, we trained the model on the relative change in blot intensity, over conditions or

O#	Species (protein)	Cell Type	Training	Prediction	Random
O1	LEF1	DLD1 CC,MDCKII,A375 MC	0.54 ± 0.167	0.505 ± 0.175	1.765 ± 0.223
O2	Vimentin	DLD1 CC,MDCKII,A375 MC	1.044 ± 0.668	0.783 ± 0.666	2.098 ± 0.784
O3	TGF β 3	DLD1 CC,MDCKII,A375 MC	0.119 ± 0.262	0.225 ± 0.418	1.408 ± 0.732
O4	E-cadherin	DLD1 CC,MDCKII,A375 MC	2.299 ± 0.449	2.154 ± 0.625	3.459 ± 0.643
O5	β -catenin	DLD1 CC,MDCKII,A375 MC	0.752 ± 0.38	0.514 ± 0.351	1.025 ± 0.0
O6	TGF β 3	DLD1 CC,MDCKII,A375 MC	1.662 ± 0.55	1.54 ± 0.677	3.328 ± 0.981
O7	GSK3-P	DLD1 CC,MDCKII,A375 MC	0.19 ± 0.291	0.203 ± 0.292	0.756 ± 0.309
O8	LEF1	DLD1 CC,MDCKII,A375 MC	0.023 ± 0.078	0.03 ± 0.11	0.937 ± 0.298
O9	E-Cadherin	DLD1 CC,MDCKII,A375 MC	1.092 ± 1.228	1.412 ± 1.348	2.652 ± 1.435
O10	Snail/Slug	DLD1 CC,MDCKII,A375 MC	0.019 ± 0.0	0.019 ± 0.0	1.111 ± 0.744
O11	LEF1	DLD1 CC,MDCKII,A375 MC	0.005 ± 0.015	0.013 ± 0.06	0.797 ± 0.431

Fig. S1: Training and prediction values as a function of condition for the 11 TGF- β objective functions versus a random parameter control.

990 time (depending upon the experiment). Thus, when using multiple data sets (possibly from
 991 different sources) that were qualitatively similar but quantitatively different e.g., slightly
 992 different blot intensities over time or condition, we captured the underlying trends in the
 993 scaled data.

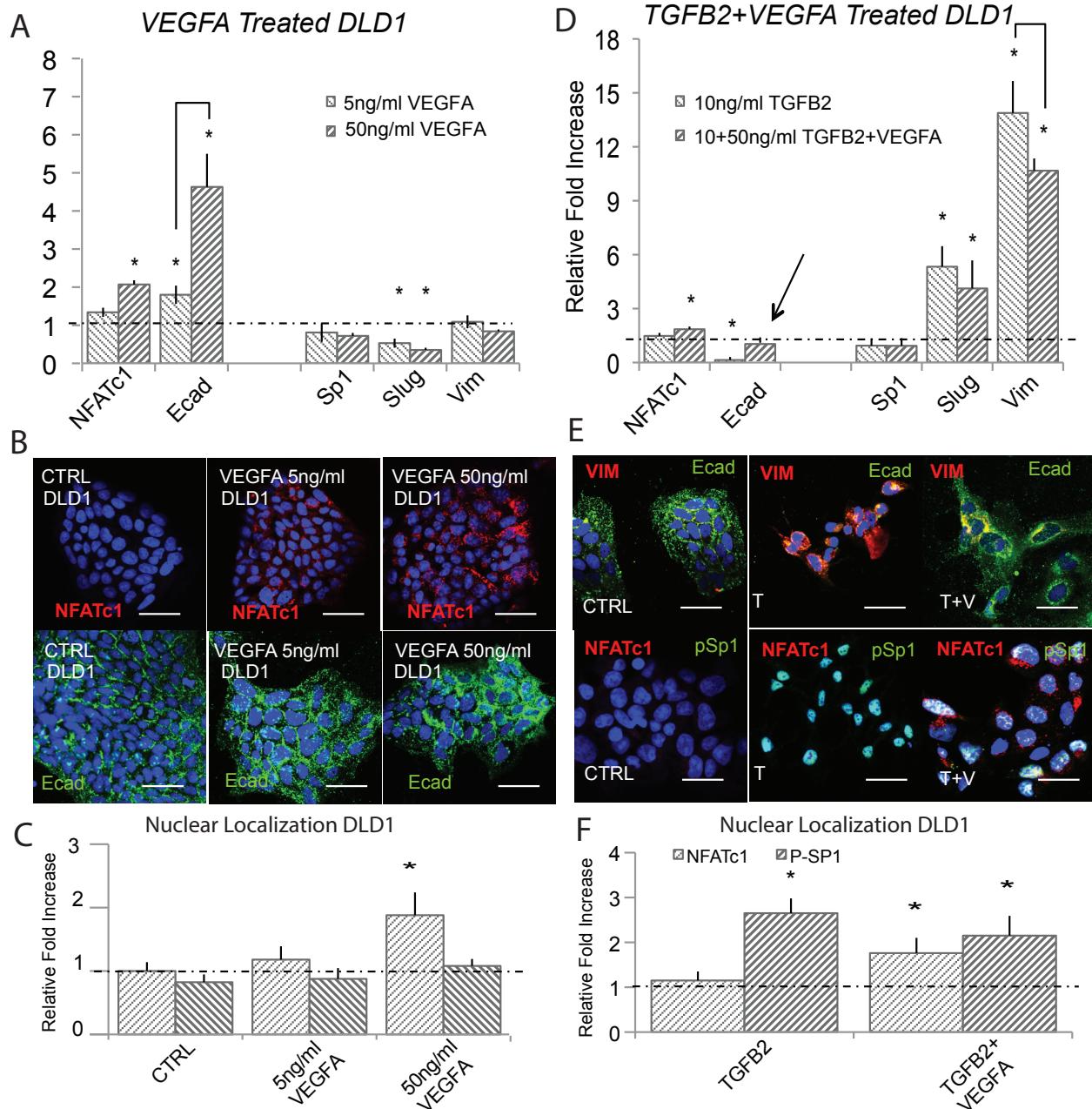


Fig. S2: VEGF-A attenuates TGF- β 1/2 to induce phenotype heterogeneity in DLD1. (A) In DLD1, we found that 5ng/ml of VEGFA increased NFATc1 and E-cadherin gene expression via qPCR and 50ng/ml potentiated this effect at 48 hrs. (B - C) These findings were confirmed at the protein level via immunofluorescence, as ecadherin levels and nuclear localization of NFATc1 increased. (D) Treatment with (10ng/ml) TGF β 2 resulted in mesenchymal transformation as measured via qPCR against target genes Slug, ecadherin, vimentin, Sp1, and NFATc1. (E - F) Immunofluorescence and nuclear localization revealed a strong presence of phospho-Sp1. (G) Combination of VEGFA (50ng/ml) and TGF β 2 (10ng/ml) treatment resulted in increased Slug, NFATc1, and vimentin expression, while also increasing ecadherin levels compared to control. (H) Immunofluorescence confirmed these results, as both ecadherin and vimentin levels were elevated. (I) A significant increase in nuclear localization of both NFATc1 and phospho-Sp1 were also found. Magnification, 40x. Scale bars: 50 μ m. C=Control, T=TGF β 2 , V=VEGFA, VI=NFAT inhibitor (VIVIT). Asterisks signify statistical differences from each other according to a one-way ANOVA with Tukey's post hoc ($p < 0.05$).

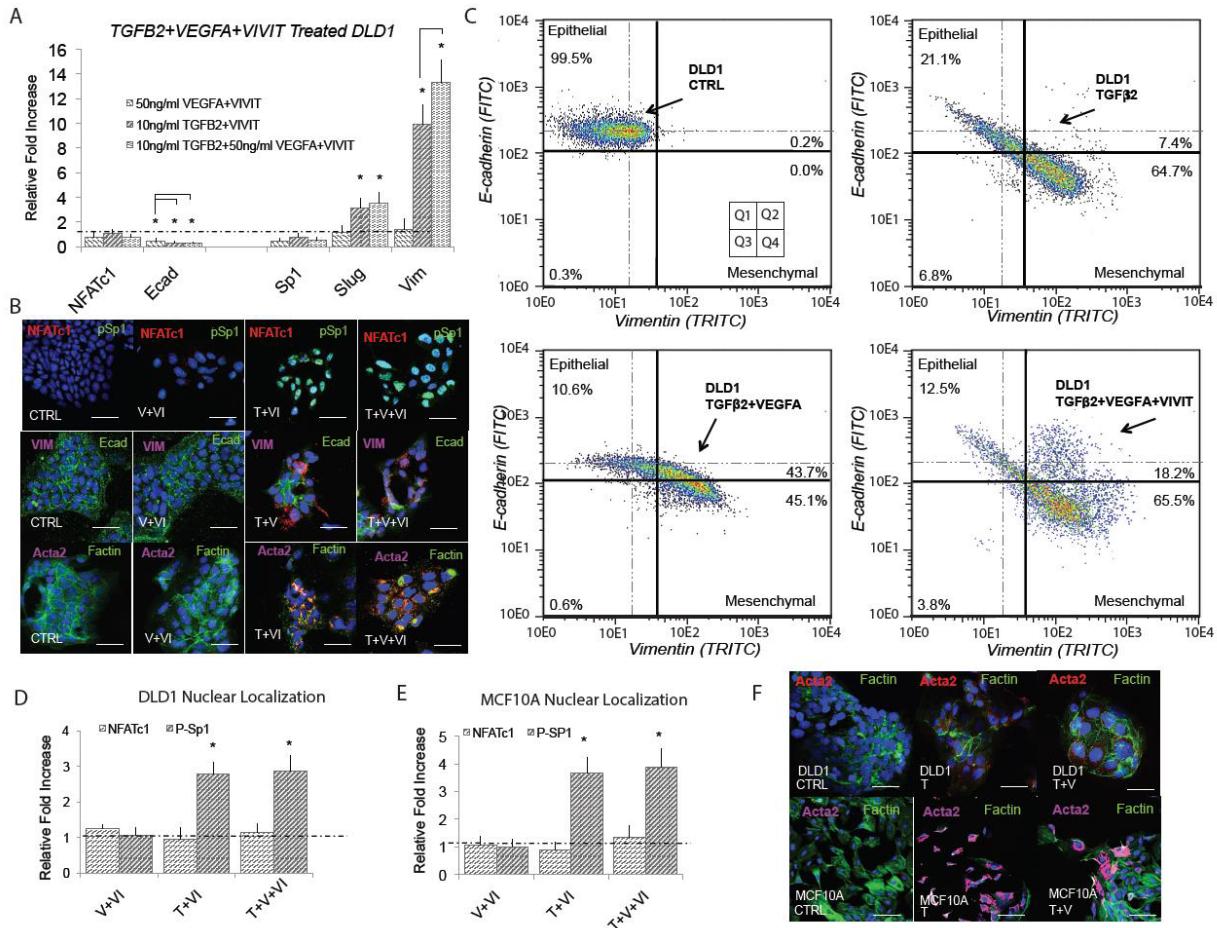


Fig. S3: E-cadherin expression is dependent upon NFAT activity in DLD1. (A) Treatment with VEGFA (50ng/ml) and NFAT inhibitory peptide VIVIT (10μM) resulted in significantly reduced ecadherin expression (qRT-PCR at 48hrs). Addition of TGF β 2 (10ng/ml) and VIVIT resulted in increased Slug and vimentin expression, while inhibiting ecadherin levels. Combined TGF β 2, VEGFA, and VIVIT treatment resulted in target genes Slug and vimentin expression increased, while inhibiting ecadherin levels. No change in Sp1 or NFATc1 expression was found. (B) These findings were confirmed via immunofluorescence as the VIVIT inhibitors was shown to inhibit ecadherin levels in all three cases. We also found no change in gene or nuclear localization of NFATc1 in all three cases, while phospho-Sp1 was found to increase in both TGF β conditions. (C) Quantitative flow cytometry also confirmed this trend. (D,E) TGF β 2, VEGFA and VIVIT treatment in DLD1 and MCF10A resulted in no change of Sp1 expression or NFATc1 expression. (F) Likewise, no change in nuclear localization of NFAT in all three cases, however phospho-Sp1 was found to increase in both TGF β conditions. Magnification, 40x. Scale bars: 50μm. C=Control, T=TGF β 2 , V=VEGFA, VI= NFAT inhibitor (VIVIT). Asterisks signify statistical differences from each other according to a one-way ANOVA with Tukey's post hoc ($p < 0.05$).

PRELIMINARY DATA USED TO HANDFIT RESPONSE OF VEGFA WITHIN SYSTEM

		MCF10A						
VEGFA		Relative mRNA			Normalized			
		Ecad	Slug	Vim	Ecad	Slug	Vim	
	5ng/ml	3HR	1.31	1.04	0.93	0.00	1.00	1.00
		48 HR	3.60	1.03	0.91	0.45	0.94	0.88
50ng/ml		3HR	1.37	0.92	0.88	0.01	0.25	0.71
		48 HR	6.34	0.88	0.76	1.00	0.00	0.00
Standard Deviation								
VEGFA		Relative mRNA			Normalized			
		Ecad	Slug	Vim	Ecad	Slug	Vim	
		5ng/ml	0.89	0.03	0.02	0.00	0.03	0.02
50ng/ml		3HR	0.78	0.03	0.27	0.10	0.03	0.26
		48 HR	0.11	0.10	0.53	0.00	0.03	0.43
VEGFA		Relative mRNA			Normalized			
		Ecad	Slug	Vim	Ecad	Slug	Vim	
5ng/ml		3HR	1.21	0.76	1.03	0.00	1.00	0.76
		48 HR	1.80	0.53	1.09	0.17	0.44	1.00
Standard Deviation								
VEGFA		Relative mRNA			Normalized			
		Ecad	Slug	Vim	Ecad	Slug	Vim	
		5ng/ml	0.80	0.10	0.18	0.00	0.13	0.13
50ng/ml		3HR	0.24	0.12	0.17	0.02	0.10	0.16
		48 HR	0.89	0.19	0.45	0.06	0.11	0.33
VEGFA		Absolute mRNA			Normalized			
		Ecad	Slug	Vim	Ecad	Slug	Vim	
5ng/ml		3HR	0.94	88.12	10.23	0.00	1.00	1.00
		48 HR	2.10	55.64	5.45	0.40	0.41	0.40
50ng/ml		3HR	1.44	64.10	8.43	0.17	0.56	0.77
		48 HR	3.85	33.40	2.32	1.00	0.00	0.00
Standard Deviation								
VEGFA		Absolute mRNA			Normalized			
		Ecad	Slug	Vim	Ecad	Slug	Vim	
		5ng/ml	0.21	22.34	2.45	0.00	0.25	0.24
50ng/ml		3HR	0.45	15.55	1.12	0.09	0.11	0.08
		48 HR	0.38	17.87	2.23	0.05	0.16	0.20
VEGFA		3HR	1.30	9.46	0.45	0.34	0.00	0.00

Fig. S4: VEGF-A qPCR data used to hand fit VEGF enhancement of E-cadherin expression. mRNA was harvested after 3hr and 24hr timepoint.