A pooled single-cell genetic screen identifies regulatory checkpoints in the continuum of the epithelial-to-mesenchymal transition

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Integrating single-cell trajectory analysis with pooled genetic screening could reveal the genetic architecture that guides cellular decisions in development and disease. We applied this paradigm to probe the genetic circuitry that controls epithelial-to-mesenchymal transition (EMT). We used single-cell RNA sequencing to profile epithelial cells undergoing a spontaneous spatially determined EMT in the presence or absence of a transforming growth factor β (TGF-β) pseudospatial trajectory model of EMT and identify regulators of its progression. These experiments uncover a hierarchy of transcription factors and cell surface receptors that drive cells through EMT. Loss-of-function of one of several surface receptors slows the progress through EMT, explaining how cells transiting through a continuous process appear to be in one of several discrete stages in some experimental systems.

Results

Pseudospatial trajectory analysis of spontaneous EMT. To define the transcriptional program executed by normal human cells undergoing EMT, we devised an in vitro system in which cells from an epithelial colony migrate into unoccupied margins of the plate (Fig. 1a). We seeded MCF10A mammary epithelial cells within cloning rings as a high-confluence patch in the center of a tissue culture dish. We then removed the rings after which cells at the border can sense adjacent unoccupied space and spontaneously undergo an EMT. The spontaneous EMT in this system is analogous to that observed for MCF10A cells on wounding in scratch-wound healing assays. Cells at the periphery of the patch acquired a spindle-like morphology and formed leading and protruding edges consistent with the acquisition of a mesenchymal phenotype (Supplementary Fig. 1). Cells collected from a single well of our assay expressed levels of E-cadherin and vimentin protein spanning a dynamic range that included those cultured at low or high confluence (Supplementary Fig. 1c,d). We dissected the patch to isolate ‘inner’ cells (2,440 cells) and ‘outer’ cells (2,564 cells). Inner and outer fractions were dissociated into single-cell suspensions and subjected to scRNA-seq on the 10x Chromium platform (Fig. 1a and Supplementary Table 1).

Unsupervised clustering with t-distributed stochastic neighbor embedding (t-SNE) separated cells from inner and outer fractions (Fig. 1b), and expression of the mesenchymal marker VIM varied in a reciprocal gradient to the epithelial markers CDH1 and DSP across embedded cells (Fig. 1c and Supplementary Fig. 2). However, we did not observe separated clusters of cells along this gradient.

During EMT, cells dissolve strong contacts and leave organized sheets, shifting from apical–basal to front–rear polarity. As they become mesenchymal, their motility and ability to break down extracellular matrix enables them to invade surrounding tissue. EMT is fundamental to development, wound healing and the metastatic dissemination of tumor cells. Several studies have identified discrete intermediate ‘stages’ of EMT based on the expression of a handful of marker genes. However, recent single-cell mass cytometry and RNA-seq analyses of breast cancer cells suggest that they fall along a continuum. As such, it remains unclear whether or not cells exist in functionally discrete states during EMT, and the genetic circuitry that controls the transition remains incompletely defined. Partial EMT is implicated in renal fibrosis and pancreatic ductal adenocarcinoma and is positively correlated with tumor grade and metastatic potential in head and neck squamous cell carcinoma (HNSCC). Characterizing the nature of intermediate EMT would have an immediate impact on our understanding of disease.

Here, we apply single-cell RNA sequencing (scRNA-seq) coupled with unsupervised machine learning techniques to analyze a pseudospatial model of EMT and identify regulators of its progression. We analyze a two-dimensional (2D) model system of spontaneous confluence–dependent EMT in human mammary epithelial cells. Cells fell continuously along a gradient of EMT progression, revealing distinct waves of gene regulation. We incorporate a pooled single-cell CRISPR-Cas9 screen into our pseudospatial trajectory analysis to define the dependency of EMT-associated signaling events on progression along the EMT continuum. These experiments uncover a hierarchy of transcription factors and cell surface receptors that drive cells through EMT. Loss-of-function of one of several surface receptors slows the progress through EMT, explaining how cells transiting through a continuous process appear to be in one of several discrete stages in some experimental systems.
axis of epithelial and mesenchymal marker expression, suggesting continual progression along an EMT rather than a sequence of discrete stages.

Individual cells at similar radii from the center of a patch could be in different stages of EMT, analogous to how cells proceed asynchronously through temporal processes such as differentiation. To resolve cellular heterogeneity and recover the program that characterizes the progress of a cell through EMT, we ordered cells using Monocle\textsuperscript{16,17}. Monocle organized cells along a linear pseudospatial\textsuperscript{14,22} trajectory, with cells from inner and outer fractions concentrated at the beginning and end of its axis, respectively (Fig. 1d and Supplementary Fig. 3). Simulated sampling from the ends of the continuum and repeating our analysis excluded the possibility that this continuity was an artifact of trajectory analysis (Supplementary Fig. 4).

Classic markers of EMT varied in expression over the trajectory. Protein and messenger RNA levels of the epithelial marker E-cadherin (CDH1) decreased as cells radiated from the center of the colony and over the pseudospatial trajectory, consistent with a spontaneous spatially determined EMT (Fig. 1e,f). Conversely, mRNA levels of VIM increased sharply in cells in the second half of the trajectory (Fig. 1f). Partial or intermediate EMT has classically been defined as the coexpression of epithelial and mesenchymal traits\textsuperscript{30}. Accordingly, cells positive for both CDH1 and VIM mRNA were most frequent in the second half of the trajectory (Supplementary Fig. 5). The population-level average expression of two epithelial markers, CDH1 and CRB3, did not vary drastically between inner and outer fractions (Supplementary Fig. 6), highlighting the value of single-cell techniques to capture the dynamics of gene regulatory changes associated with EMT.

We next identified genes regulated during EMT by performing differential expression analysis parameterized by the position of each cell along the trajectory (Supplementary Table 2). Clustering the 1,105 differentially expressed genes (DEGs) (likelihood ratio test; false discovery rate (FDR) < 1 × 10\textsuperscript{-10}; area under the curve (AUC) > 10 in at least one quantile, see Methods and Supplementary Table 3) revealed six groups of genes with similar kinetics. We performed geneset analysis using the Gene Ontology biological processes\textsuperscript{13,22} and MSigDB hallmarks molecular signature\textsuperscript{21} geneset collections. Genes in cluster 6 were upregulated and enriched for roles in translational regulation or EMT, while those in downregulated cluster 1 were linked to epithemis development. Cluster 5, highly expressed in the outermost regions of the pseudospatial trajectory, was associated with the regulation of the cell cycle, consistent with the relief of contact-mediated inhibition of proliferation (Fig. 1g,h and Supplementary Table 4).

Geneset analysis identified pathways upstream of pseudospace-dependent gene expression. Cluster 1 was enriched for genes repressed by active KRAS signaling\textsuperscript{22,29}, including some with roles in EMT. For example, keratin 1 (KRT1) was expressed in cells at the epithelial end of the trajectory but silenced as cells approached the border of the patch (Supplementary Fig. 7). Keratin\textsuperscript{21} traffic E-cadherin to the cell membrane, while vimentin does not\textsuperscript{30}, and the shift in cytoskeletal filament composition from keratin- to vimentin-containing is integral to EMT\textsuperscript{34}. The EMT-associated cluster 6 included the unfolded protein response (UPR) transcriptional regulator ATF4 whose increased expression preceded upregulation of genes in cluster 5, which was enriched for genes associated with the UPR (cluster 5 and 6, Fig. 1g,h and Supplementary Fig. 8), consistent with a recent study demonstrating that the induction of EMT elicits protective activation of the UPR\textsuperscript{22}.

Repeating our spatial EMT assay and single-cell transcriptional profiling using primary human mammary epithelial cells (HuMEC) identified a similar linear pseudospatial trajectory and distribution of inner and outer cells (Supplementary Fig. 9a,b and Supplementary Table 5). The dynamics of epithelial and mesenchymal marker expression was comparable albeit with decreased CDH1 downregulation and more drastic upregulation of FN1 (Fig. 1i and Supplementary Fig. 9c). Having identified a spatial EMT in another epithelial cell type we sought to understand how this phenotype changes in response to a strong inducer of EMT.

**Pseudospatial trajectory alignment elucidates transforming growth factor β (TGF-β)-driven full EMT.** Activation of the TGF-β pathway leads to a powerful induction of EMT\textsuperscript{33,34}. We repeated our pseudospace experiment, this time treating cells with TGF-β to promote mesenchymal conversion in MCF10A cells. We sequenced transcriptomes of 2,121 inner and 2,116 outer colony cells that were segregated in t-SNE space but did not form coherent clusters, and whose expressed FN1 and VIM continuously varied (Supplementary Fig. 10). Thus, adding a strong extracellular signal promoting EMT did not drive cells into discrete stages. We therefore constructed a pseudospatial trajectory for TGF-β as well (Supplementary Fig. 11).

To compare cells from spontaneous and TGF-β-driven EMT trajectories, we used trajectory alignment\textsuperscript{13,22} using Dynamic Time Warping\textsuperscript{35} to map cells onto a common pseudospatial axis (Fig. 2a). Along the aligned axis, CDH1 and CRB3 were expressed in cells treated with TGF-β with similar kinetics to those undergoing confluence-mediated EMT (Fig. 2b), and consistent with reports that maintenance of cell–cell contacts prevents TGF-β stimulation from fully repressing an epithelial phenotype\textsuperscript{16}. However, TGF-β exposure is sufficient to drive the expression of mesenchymal genes even in cells within the epithelial core. Additionally, only cells treated with TGF-β and positioned at the outer extreme of the trajectory expressed robust levels of FN1 and CDH2, suggesting a full E- to N-cadherin switch. Exposure of HuMEC cells to TGF-β similarly led to a robust increase in VIM and FN1 at the beginning of the trajectory (Supplementary Fig. 12c); however, expression of CDH2 was not apparent. A broader geneset analysis comparing normalized average expression scores\textsuperscript{3} showed that TGF-β drove MSigDB Hallmark EMT genes higher and Gene Ontology biological process epithemis development genes lower in both MCF10A and HuMEC cells (Supplementary Fig. 13).

To identify genes responsive to TGF-β, we tested for differential expression as a function of TGF-β treatment, subtracting changes attributable to pseudospatial position. This analysis identified 1,328 genes in 10 clusters with distinct TGF-β-dependent dynamics (Fig. 2c, likelihood ratio test; FDR < 1 × 10\textsuperscript{-10} and |ΔAUC| > 0.02, see Methods, Supplementary Fig. 14 and Supplementary Table 6). For example, cluster 5 contained cell-cycle-related genes upregulated along both trajectories (Fig. 2c and Supplementary Table 7). Cluster 4 contained genes upregulated toward the end of the spontaneous trajectory but maintained at high levels throughout the TGF-β-mediated trajectory (Fig. 2c). This cluster included two EMT-associated genes, one of which, \textit{NNMT}, is a marker of the metabolic changes that accompany EMT\textsuperscript{44} (Fig. 2d). In contrast, clusters 6 and 8 contained EMT genes that peaked at the middle or end of the TGF-β-driven trajectory, respectively (Fig. 2c,e,f), but were unaltered or induced to a lesser degree in the spontaneous trajectory. Therefore, cells at comparable positions in spontaneous versus TGF-β-mediated EMT continua as defined by epithelial markers display pronounced transcriptional differences.

To explore which molecular regulators are responsible for shared and distinct patterns of spontaneous and TGF-β-mediated gene regulation during EMT we performed geneset analysis using the MSigDB Oncogenic Signature geneset collection. This geneset collection is composed of genes whose expression increases or decreases as a function of perturbing signaling pathways\textsuperscript{18}. Cluster 8 included genes upregulated as cells treated with TGF-β undergo EMT but are weakly altered during spontaneous EMT. These were enriched for genes expressed in response to KRAS signaling\textsuperscript{30}, including genes with roles in EMT, such as \textit{CXCLI} and \textit{CXCL2}, which induce
cellular migration. Conversely, cluster 10 included epithelial marker genes downregulated early in spontaneous EMT and expressed at low levels in cells treated with TGF-β (for example KRT4 and KRT16) (Supplementary Fig. 16). These and several others are known to be repressed by active KRAS signaling. This observation, together with pathway analysis of spontaneous...
EMT, implies that KRAS signaling is sustained throughout both spontaneous and TGF-β-driven transitions, suggesting it governs multiple points of the EMT continuum in normal cells.

Single-cell flow cytometric profiling of TGF-β-induced EMT described the transition as a three-state process7,46. In contrast to this 'discrete' view, we observed a continuous trajectory over which cells were distributed and along which many genes, including classic markers of epithelial and mesenchymal states, exhibit smooth changes in expression. Few cells undergoing spontaneous EMT expressed high levels of some mesenchymal markers, raising the possibility that we failed to capture some discrete, physiologically important 'stages' of EMT. However, exposing cells to TGF-β also drives them over a continuum, albeit one with different spatial patterns of transcriptional regulation.

To investigate whether tumor cells in vivo transit through an EMT continuum similar to the one observed in vitro, we re-analyzed scRNA-seq data from patients with HNSCC15. The most mesenchymal tumor, as ranked by Puram et al.15, expressed EMT genes at similar levels to cells at the outer end of our TGF-β-driven pseudospatial trajectory (Supplementary Fig. 17). Genes that make up early and late waves of KRAS-associated EMT in vitro (cluster 10 and 8, respectively, Fig. 2c) were expressed in a manner consistent with their partial EMT phenotypes assigned by Puram et al. (Fig. 2g,h). To confirm that the similarity between our in vitro model and the tumor cells was not limited to known EMT genes, we projected tumor cells onto our spontaneous and TGF-β-driven trajectories based on full transcriptome signatures using a nearest-neighbor matching algorithm5 (Methods). Most tumor cells mapped to the end of our spontaneous EMT trajectory. In contrast, tumor cells projected more uniformly over the TGF-β-mapped to the end of our spontaneous EMT trajectory. In contrast, cells were distributed and along which many genes, including clas 

A pooled loss-of-function screen identifies genes regulating EMT progression. We reasoned that certain regulators control passage through parts of the EMT continuum and a lack of one or more of these signals leads to accumulation of cells at 'discrete' EMT 'stages'. To identify regulators of progression along the continuum, we devised a high-throughput loss-of-function screen to probe the architecture of pathways with known involvement in EMT. Several groups recently devised methods for coupling CRISPR-based screens and a scRNA-seq readout, thereby capturing the identity of the single-guide RNA(s) (sgRNAs) that a cell received in conjunction with its gene expression profile49-52. Here we used a modified version of CRISPR droplet sequencing (CROP-seq)52, which does not rely on the pairing of sgRNAs with distally located barcodes. We recently showed that this design is preferable to alternatives, avoiding template switching between sgRNAs and associated barcodes during lentiviral co-packaging41.

We selected 16 cell surface receptors and 24 transcription factors for screening via CROP-seq in our 2D EMT system (Fig. 3a). These targets include receptors reported to activate KRAS (EGFR, MET, FGFR1, FGFR2, ITGA5, ITGB1 and ITGB3)13-17 along with others that drive Wnt, Notch and other pathways (Fig. 3b). Transcription factors that activate or repress EMT genes included both well-characterized (SNAI1/2, TWIST1/2 and ZEB1/2) and recently reported (FOXD3, GATA6 and SOX9) regulators1. We repeated our in vitro EMT assay with a mixture of cells edited with sgRNAs to one of the 40 genes (or non-targeting controls, NTC) and subjected them to scRNA-seq after being cultured with TGF-β (12,337 cells) or without (17,093 cells). Unsupervised clustering analysis of cells treated with TGF-β identified prominent, clearly demarcated clusters of cells that retained expression of the epithelial markers CDH1 and CRB3 and failed to upregulate FNI and VIM (Fig. 3c and Supplementary Fig. 19,a,b). Cells expressing sgRNAs targeting TGFBR1 and TGFBR2 were enriched in these clusters (Fisher’s exact test; FDR < 1 × 10−9) (Fig. 3d,e and Supplementary Fig. 19c), while NTC sgRNAs were largely absent from them. Importantly, this distribution was not caused by the number of TGFBR1 and TGFBR2 sgRNA cells in our screen (Fig. 3f). Cells with sgRNAs against TGFBR1 and TGFBR2 expressed lower levels of FNI and VIM than those with NTC sgRNAs, indicating a failure to activate a TGF-β-driven EMT (Fig. 3g) and confirming that CROP-seq can be used to identify molecular trajectories along the EMT continuum.

We next sought to organize edited cells into a pseudospatial trajectory. We compared NTC cells from inner and outer fractions, which revealed 1,197 and 761 DEGs in the spontaneous and TGF-β-driven EMT, respectively, more than 80% of which were also found in unedited EMT experiments (Supplementary Fig. 20a–d). Pseudospatial trajectories reconstructed from NTC cells aligned to unedited trajectories with only minimal warping (Supplementary Fig. 20e–g). We then provided Monocle2 with all edited cells, which constructed trajectories along which EMT marker genes were expressed with kinetics similar to unedited cells (Supplementary Fig. 21). Differential expression analysis identified 978 and 4,079 genes that varied across genotypes along spontaneous and TGF-β-driven trajectories, respectively (Supplementary Fig. 22 and Supplementary Tables 8 and 9).

We hypothesized that loss of surface receptors that transduce signals important for EMT, or the transcription factors they drive, would alter a cell's progress along the trajectories. To determine whether loss-of-function of EMT-associated targets altered their progression along pseudospace, we divided the trajectory into bins according to the density of cells along spontaneous and
TGF-β-driven EMT trajectories resulting in 7 and 8 bins, respectively. We then tested whether cells carrying sgRNAs against each target altered their distribution over these 'regions' of the aligned trajectories, relative to NTCs. We determined empirical false-discovery rates of these tests by comparing enrichments of knockout cells to a random sampling of NTC cells (Supplementary Fig. 23, see Methods for details).

Of the 40 genes tested, 30 significantly shifted the pseudospatial positions of the cells when targeted via CROP-seq, with 11 overlapping between conditions (Fig. 4a,b and Supplementary Fig. 23).
Some targets were modestly enriched (less than two-fold) at a given pseudospatial position. For example, in the spontaneous EMT trajectory, cells with sgRNAs targeting FZD7 were enriched at region 1, near the epithelial core of the trajectory, and region 3 (Fig. 4a). Other gene knockouts induced strong, focal accumulation of cells at one or two positions along the EMT continuum (Fig. 4a,b). Loss of EGFR induced focal accumulation at region 3 (Supplementary Fig. 24a). Similarly, cells with sgRNAs against MET were enriched in regions 2 and more strongly in region 3. The majority of significantly enriched targets accumulated in region 3 directly preceding a decrease in the total number of CDH1 single-positive cells and an increase in CDH1/VIM double-positive cells (Supplementary Fig. 25).

Edited cells across the TGF-β-treated trajectory had a distinct set of genes from those that control progression through spontaneous EMT, reflecting the direct activation of EMT-associated transcription factors by SMAD signaling58. The pseudospatial regions encompassing the first half of the trajectory were strongly enriched for TGFBR1 and TGFBR2 knockouts (region 1–4, Fig. 4b).
Fig. 4 | Accumulation of knockout cells across spontaneous and TGF-β-driven EMT trajectories identifies regulators of discrete checkpoints across the EMT continuum. a, b, Enrichment of knockouts whose distribution is significantly altered across pseudospace, and therefore EMT progression, in our spontaneous (11,908 cells) (a) and TGF-β-driven (9,951 cells) (b) conditions. The distribution of cells expressing sgRNAs against EMT genes was compared to the distribution of NTC controls by using chi-square (empirically determined FDR < 10%). For targets whose distribution is altered enrichment across each region was determined by calculating the odds ratio. c, Percentage of E-cadherin (top panels) or vimentin (bottom panels) positive cells in MCF10A colonies exposed to MEK (U0126) and PI3K (L Y294002) inhibition after spontaneous (left panels) or TGF-β-driven (right panels) EMT. Error bars denote standard deviation from the mean (n = 3, two-tailed Student’s t-test). d, Percentage of E-cadherin (top panels) or vimentin (bottom panels) positive cells in MCF10A colonies exposed to EGFR (Erlotinib), MET (Crizotinib), FGFR (Infigratinib) and ITGAV (Cilengitide) inhibition after spontaneous (left panels) or TGF-β-driven (right panels) EMT. Error bars denote standard deviation from the mean (left, spontaneous EMT control/EGFRi/ITGAVi n = 7, METi/FGFRi n = 4 independent samples; at right: TGF-β-driven EMT control n = 4, EGFRi/METi/FGFRi/ITGAVi n = 3 independent samples, two-tailed Student’s t-test). e, Inferred EMT regulatory network and putative regulators identified in this study. f, Model depicting the MEK dependent EMT regulatory checkpoint created and its effects on the development of intermediate EMT phenotypes.
and Supplementary Fig. 24b). As in spontaneous EMT, the loss of numerous genes in TGF-β-treated cells concentrated them at defined pseudospatial positions. ZEB1, proposed to effect an irreversible switch to an epithelial mesenchymal state,\textsuperscript{7} GATA6, NOTCH1 and POU5F1 were concentrated beginning in region 3, suggesting that this position in the trajectory coincides with a decision point cells pass through during EMT.

Of the seven receptors in our screen known to activate Ras/MAPK signaling, five (EGFR, MET, ITGAV, ITGB1 and FGFR1) altered the distribution of cells over the trajectory, and all but MET concentrated them at just one or two regions. Interestingly, only MET and ITGAV did so during spontaneous and TGF-β-driven EMT. In the spontaneous EMT trajectory, early accumulation of cells expressing sgRNAs against the receptor tyrosine kinases EGFR and MET\textsuperscript{5,6}, suggested that one or both are responsible for the early wave of KRAS activity associated with exit from the epithelial state. In the TGF-β-mediated EMT trajectory regions 3 and 4 displayed a robust accumulation of cells expressing sgRNAs against the ITGAV integrin and regions 1, 5 and 7 were enriched for cells expressing sgRNAs against the tyrosine kinase FGFR1\textsuperscript{10}. Integrins function as heterodimers between α and β subunits and αvβ1 heterodimers have been shown to mediate TGF-β signaling during fibrosis,\textsuperscript{14} a process where EMT has an important role\textsuperscript{1,15,16}. These precede the terminal EMT state in our TGF-β trajectory and may contribute to the KRAS-associated late EMT signature identified by our dynamic time warping analysis (Fig. 2c).

To understand how KRAS signaling drives cells through EMT, we performed an in vitro assay in the presence of small molecules that block Ras signaling. Ras exerts its regulatory program via activation of the RAS/RAF/MEK/ERK and PI3K/AKT pathways.\textsuperscript{17,18} We therefore tested whether loss of MEK (via U0126 treatment) or PI3K signaling (via LY294002 treatment) is sufficient to block the exit from the epithelial state and/or acquisition of mesenchymal phenotypes. Doses of both drugs were chosen to minimize effect on the proportion of cells expressing vimentin, N-cadherin or cytoplasmic fibronectin, protein (Fig. 4c and Supplementary Fig. 29). This suggests that activation of the RAF/MEK/ERK pathway is required for the downregulation of the epithelial program in normal mammary epithelial cells, but that alternate pathways can activate the mesenchymal program when RAF/MEK/ERK signaling is blocked.

Lastly, we explored how the expression of factors that alter the accumulation along EMT in MCF10A relate to the diverse EMT phenotypes observed in HNSCC tumors. Hierarchical clustering of the mean expression level of cell surface receptors identified a strong relationship between receptor expression and the extent of EMT across tumor samples (Supplementary Fig. 30). Expression of FZD2, FZD7, FGFR1 and PTC1\textsuperscript{19} was inversely correlated with levels of EMT genes. With the exception of PTC1, edited cells lacking these genes were enriched at the beginning of our EMT trajectories (Supplementary Fig. 30). Conversely, tumors expressing high levels of EMT genes (Supplementary Fig. 30) also expressed MET, ITGAV, ITGB1, TGFBR1 and TGFBR2.

Discussion

The integration of single-cell trajectory analysis and pooled genetic screening has the potential to map the genetic circuits that control progression across biological transitions. Understanding the regulation of EMT is a fundamental goal in developmental and cancer biology and has the potential to yield new therapeutic opportunities for intervention in cancer. In contrast to numerous reports of ‘partial,’ ‘hybrid’ or ‘intermediate’ EMT stages, both our analysis and recent scRNA-seq and mass cytometry studies of a cancer line\textsuperscript{20,21} indicate that cells are organized along a continuum during EMT.

Our CRISPR/scRNA-seq loss-of-function screen reconciles these two conflicting views of gene regulation in EMT. Previously, we showed that a loss-of-function mutation can create a branch from the wild-type trajectory by which cells execute an alternative gene expression program\textsuperscript{22}. Here, we show that transcription factor and signaling receptor gene knockouts can cause cells to accumulate at defined points along the EMT continuum, implying the existence of a sequence of ‘checkpoints’ to progress through it. Therefore, although cells fall along a transcriptional continuum during EMT, genetic insults that disable key signaling pathways could enrich a particular gene expression profile within a cell population, creating the impression of a stable intermediate phenotype. Consistent with this finding, recent single-cell profiling of HNSCC found evidence for diverse partial EMT states at the leading edge of tumors\textsuperscript{23}, which could arise from genetic heterogeneity amongst cancer cells. Our analysis suggests that local variation in signaling in key pathways could also contribute substantially to the EMT phenotype of a tumor.

Several large modules of genes with distinct wave-like patterns of regulation during spontaneous- or TGF-β-mediated EMT were enriched for targets of KRAS, which may, therefore, be involved throughout the EMT continuum. KRAS signaling can be initiated via various upstream signals, making it difficult to pinpoint the signaling that is driven at each point on the continuum. Focal accumulation of cells lacking particular effectors of KRAS signaling early in spontaneous (EGFR and MET) and late in TGF-β-mediated (FGFR2 and ITGAV) EMT suggests that the cell responds to a sequence of cues to execute steps in the program. TGF-β and RAF/MEK/ERK
are known to be involved in EMT, but how the two pathways interact during the process is not clear. Here, we show that in the absence of exogenous TGF-β, inhibiting RAF/MEK/ERK is sufficient to block exit from the epithelial state and prevent activation of the mesenchymal gene expression program (Fig. 4e). However, when cells are exposed to exogenous TGF-β, this pathway can ‘shortcut’ MEK to activate the mesenchymal program directly. Further, we find that loss of MEK activity can lock cells in a partial EMT-like state where cells coexpress E-cadherin and high levels of early and late mesenchymal markers. Taken together, these observations point to the existence of ‘checkpoints’ in the EMT continuum at which cells can arrest and accumulate, creating the impression of discrete stages in bulk cell assays (Fig. 4f).

Our study combines single-cell trajectory analysis with high-throughput pooled loss-of-function screening, which constitutes a powerful approach for identifying upstream signals of pathways that regulate cellular phenotypes. We expect that this methodology will shed light on the genetic architecture that governs not just EMT but diverse biological processes in development and disease. More generally, the observation that interrupting a signaling pathway can enrich a particular transcriptional state within a cell population will inform ongoing debates surrounding the definitions of cell type and state and the delineation of human cellular ontology.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41588-019-0489-5.

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Author contributions

J.L.M.F., J.S. and C.T. devised the project. J.L.M.F., A.J.H., J.S. and C.T. designed experiments. J.L.M.F., A.J.H. and D.J. performed experiments. D.J. and X.Q. provided substantial technical and computational support, respectively. J.L.M.F. and A.J.H. performed analyses. J.L.M.F. and C.T. wrote the manuscript with the support of the other authors.

Competing interests

The authors declare no competing interests.

Additional information

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Methods
Cell culture. MCF10A breast epithelial cells were purchased from ATCC and used within ten passages. HuMEC were purchased from ThermoFisher Scientific and passage 4 cells were used for all experiments. Cas9-expressing MCF10A (MCF10A-Cas9) were generated by transduction with lentCas9 blasticidin (Addgene) and selected with 10 μg/ml blasticidin (ThermoFisher Scientific) 72 h post-transduction. Cells were cultured at 37°C and 5% CO2 in MCF10A media composed of DMEM/F12 (ThermoFisher Scientific) containing 10% fetal bovine serum (ThermoFisher Scientific), 1% Pen-Strep (ThermoFisher Scientific), 10 μg/ml penicillin and 10 μg/ml streptomycin.

For E-cadherin pure ethanol for 10 min at room temperature. For crystal violet staining, fixed the previously marked cloning ring and wells were inspected under a dissecting microscope to assess the purity of the fraction.

Flow cytometry for EMT marker protein levels. MCF10A cells were plated at the center of wells in six-well plates as previously described. Two hours after plating, the cells were harvested using TrypLE, washed twice with PBS, and resuspended in 500 μl of PBS, fixed for 1 h at room temperature, and blocked with 1% BSA in PBS. Fixed samples were split into two and one aliquot incubated overnight with mouse anti-cytokeratin 8/18 (Cell Signaling) for 2 h at room temperature and washed with 1% BSA in PBS. For imaging, colonies were incubated for 1 h in IF buffer containing Alex-488 conjugated goat anti-mouse IgG in IF buffer (Invitrogen), washed with IF buffer and 5 μg/ml Hoechst 33342 (Invitrogen) added to colonies. Brightfield imaging of crystal violet-stained whole colonies was performed on a Zeiss Axio Observer Z1 microscope.

To extract and visualize spatially dependent genes. Trajectories were constructed according to the procedure recommended in the Monocle2 documentation (http://cole-trapnell-lab.github.io/monocle-release/docs/#constructing-single-cell-trajectories). Briefly, genes used to order cells were selected by comparing the inner and outer cell fractions in the assay. For each cell specifically: 47,905 and 30,636 mean reads per cell for initial MCF10A and HuMEC pseudospatial experiments, respectively, and 43,557 mean reads per cell for CROP-seq experiments. Additional metrics for each individual scRNA-seq library can be found in Supplementary Table 1 and Supplementary Fig. S31.

t-SNE. We performed principal component analysis (PCA) on a matrix composed of cells and gene expression values for genes expressed in more than 50 cells, reduced dimensions to the top 25 principal components and a t-SNE initialization in this PCA space to reduce to 2-t-SNE dimensions using the reduced dimension function in Monocle2 specifying num_dim = 25, max_component = 2, norm_method = log and reduction_method = t-SNE. To visualize the gene expression levels of EMT markers in t-SNE space the gene expression levels of CDH1, DSP and VIM in every cell were normalized by the library size of each cell (the Size_Factor in Monocle2), a pseudocount of 0.1 was added and values log2 normalized.

Pseudospatial reconstruction of single-cell transcriptomes. Trajectories were constructed according to the procedure recommended in the Monocle2 documentation (http://cole-trapnell-lab.github.io/monocle-release/docs/#constructing-single-cell-trajectories). Briefly, genes used to order cells were selected by comparing the inner and outer cell fractions in the assay. For each cell specifically: 47,905 and 30,636 mean reads per cell for initial MCF10A and HuMEC pseudospatial experiments, respectively, and 43,557 mean reads per cell for CROP-seq experiments. Additional metrics for each individual scRNA-seq library can be found in Supplementary Table 1 and Supplementary Fig. S31.

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Detection and visualization of spatially dependent genes. To extract and visualize genes that vary over a trajectory (beyond the variability one would expect across unsorted cells), we used the procedure recommended by the Monocle2 documentation (http://cole-trapnell-lab.github.io/monocle-release/docs/#inducing-genes-that-change-as-a-function-of-pseudotime). To identify changes in gene expression across pseudospatial trajectories we fit splines with three degrees of freedom to capture the dynamics of gene expression over pseudospace and tested for differential gene expression analysis using a full model of y ~ sm.ns (pseudospace, df = 3), which encode the position of a cell on the trajectory as a continuous covariate. To further filter genes by those with the largest effect size we divided pseudospace into five quantiles, calculated the AUC for each gene at each quantile and filtered DEGs to those having an AUC > 10 in at least one quantile and an FDR of FDR < 10−10. DEGs were variance stabilized and scaled, clustered and visualized using the heatmap function from the R package heatmap specifying
ward.D2 as the clustering method. To identify biological processes and pathways enriched in clusters of DEGs across pseudospase we performed hypergeometric testing using the piano R package specifying genes expressed in more than 50 cells as the background set.

Calculation of aggregate gene expression scores. To determine the extent to which cells in different samples activate certain gene expression modules we calculated a normalized aggregate expression score for each cell for defined genesets. For a matrix of genes and cells, log2 normalizing gene expression was defined for genes in each set after library size normalization and addition of a pseudocount of 1. For each cell, we then calculated the mean normalized expression level of genes in the geneset and mean-centered and variance scaled mean normalized expression values across all cells. The compare_means function from the ggpubr package was used to determine the significance in changes of scores between endpoints in MCF10A and HuMEC EM trajectories (Supplementary Fig. 13) specifying the wilcox.test as the method and using the Holm procedure (holm) to correct for multiple hypothesis testing.

Dynamic time warping of pseudospatial trajectories. Alignment of Mock and TGF-β-driven trajectories for MCF10A, MCF10A-Ca9 and HuMEC pseudospatial trajectories was performed as described35, setting the Mock and TGF-β-treated cell trajectories as the reference and query, respectively. Briefly, to arrive at a common pseudospatial axis, trajectories were aligned based on the intersect of genes used for ordering Mock and TGF-β-driven trajectories where the final alignment were scaled from 0-100. splines were fitted to each gene using the geneSmoothCurves function in Monocle2, splines were variance stabilized and scaled before alignment using the dtw function from the DTW R package using the following options: step pattern = ’rbaber’ Juang step pattern (type = 3 and slope.weighting = c), open.begin and open.end = FALSE. To identify genes that describe the differences in the interaction between pseudospaces and TGF-β treatment across Mock and TGF-β-driven trajectories we performed differential gene expression analysis using a full model of “y ~ pseudospace*treatment’ and a reduced model of “y ~ pseudospace”. We isolated DEGs with the largest differences between treatments by dividing pseudospace into five equally spaced quantiles, calculating the AUC (calculated using spline interpolation) for each treatment within each quantile and identifying genes with a relative difference in AUC (relative AUC difference = abs(AUC1 − AUC2)/sum(AUC1 + AUC2)) larger than 0.02 in at least one quantile and an FDR < 1 x 10−10.

Preprocessing of the HNSCC dataset. Processed data from the scRNA-seq of HNSCC tumors described in Puram et al.34 were downloaded from the GE2 Omnibus database (GSE103322) and a Monocle2 Dataset (cds) object was created using gene expression and metadata available in GSE103322. HNSCC trajectories were set as alignment genes and the open.begin and open.end parameters of the dtw function in the DTW R package was set to TRUE to allow alignment of HNSCC tumor samples anywhere along the MCF10A trajectories. The dtwPlot function from the DTW R package was used to visualize the alignment of HNSCC trajectories to either MCF10A spontaneous or TGF-β-driven EM trajectories.

Cloning, lentiviral packaging and transduction of CROP-seq libraries. CROP-seq lentivector (Addgene) was prepped for sgRNA library insertion as described33. Briefly, vector was digested using BsmBI (New England Biolabs) and fast alkaline phosphatase (ThermoFisher Scientific). Oligonucleotides (IDT), each containing an sgRNA and homology for Gibson ligation, were designed as follows: [U6 homology]-[sgRNA]-[sgRNA backbone homology]

5′-tacctGTTGAGGAGCGAAACACC[G]-[20 bp sgRNA]−3′aggagagtGAA Atagaagtaaataagg-3′ where the addition of the G immediately upstream of the sgRNA ensures transcription from pol III promoters. Oligonucleotides (overall design and individual sgRNA sequences can be found in Supplementary Table 10) were made double-stranded by PCR with primers against the invariant regions. The digested CROP-seq vector (10 fmols) and 200 fmols of double-stranded oligonucleotides were ligated using the In-Fusion HD kit (Clontech) by incubation at 50°C for 1 h. Libraries were then transformed into stellar competent cells (Clontech), transformations were diluted in 250 μL of LB, spread onto 6 LB agar plates containing ampicillin and bacteria culture at 30°C for 24h. Resulting colonies were scraped with LB, pooled and vector recovered using a DNA midi kit (Qagen). Lentivirus was generated by transfecting HEK293T in MCF10A media lacking Pen-Strep with our CROP-seq vector using the ViraPower lentiviral packaging mix (ThermoFisher Scientific) according to manufacturer’s instructions. Lentivirus-containing supernatant was filtered using a 45μm filter (ThermoFisher Scientific), MCF10A-Ca9 cells were transduced with increasing amounts of the CROP-seq lentiviral library and selected with puromycin, retaining transduced cells that had an approximate multiplicity of infection of 0.3.

Enrichment of sgRNA containing transcripts and genotype assignment. For CROP-seq experiments, a nested PCR was performed on 5–10ng of unheated cDNA to enrich for sgRNAs positioned on the 3′ UTR of the puromycin resistance gene transcripts. All oligonucleotide sequences used for enrichment of sgRNA containing transcripts can be found in Supplementary Table 10. Briefly, PCR reactions were performed using a 27 reverse primer primer equivalent to the one introduced by the oligo containing beads in the 10X Chromium Single-cell 3′ solution V1 (5′-CAAGCGAAGAGCGGATACAG-3′). For the first PCR, the forward primer directed toward the beginning of the U6 promoter was 5′-TTTCCCATATGTTCTCATATTTGC-3′. For the second PCR, the forward primer binds at the beginning of the sgRNA and adds the standard Nextera R1 sequence: 5′-TGTCGGCGACGGTCGATGTGATAGAGACACGCGTTGGAAGACGGAAACAC-3′. In the final PCR, amplicons were indexed with standard Nextera R5 indexers: 5′-ATGATACGGCCACGACCCGAGATCTACAC[10bp Index]-TGTCGGCGACGCCTC-3′. A 1X Ampure cleanup was performed after each PCR. A fifth of PCR1 was added to PCR2 and a 25th of PCR2 was added to PCR3. Libraries were sequenced as spike-ins with transcriptome scRNA-Seq libraries. Final cellular barcodes and UMI were extracted from position sorted BAM file output by Cell Ranger 1.3.1. We then attempted to find a perfect match for sequences preceding the sgRNA (GTGAAAGGACGAAAAACGC) or used a stripped Smith–Waternon alignment to locate the sequence within an error tolerance of 2 bp shorter than the expected sequence. For each match or alignment, the sgRNA sequence is extracted and compared to a whitelist of all sgRNA within an edit distance of half the minimum distance between any pair of guides in our scRNA library tracking matches for each cell. Cimeric sequences were removed by the approach as detailed in a previous report44. sgRNA sequences with over three reads accounting for more than 7.5% of sgRNA reads assigned to a given cell were assigned to each cell. These assignments were combined with the filtered gene expression matrix created by Cell Ranger to assign high-quality cells.

t-SNE and distribution of knockout cells across PCA space. We performed PCA on a matrix composed of cells each containing only one guide from our CROP-seq screen and gene expression values for genes expressed in more than 50 cells and reduced dimensions to 25 principal components. t-SNE was initialized in this PCA space to reduce to two t-SNE dimensions. We then performed louvain clustering across PCA space. A chi-square test was performed to determine whether the distribution of a sgRNA and targets in PCA was significantly different compared to NTC at an FDR cutoff of 5%. Knockouts whose distribution was significantly different from NTC were subjected to further analysis. For each sgRNA we derived a functional editing rate using an expectation maximization approach by first modeling the PCA distribution as a mixture of cells with functional and non-functional edits where the mixing parameter is the relative functional edit rate for the sgRNA; estimating the weighted average of the empirical PCA distribution for each guide; and estimating relative functional edit rate as the one that maximizes the observed PCA distribution. Weighted
We then used these guide RNA weights to arrive at weighted cell counts of guide RNA expressing a guide RNA across pseudospatial regions under the mixture model. For the maximization step, we chose the relative function edit rate for a guide RNA as that which maximizes the likelihood of the observed distribution of cells expressing non-targeting control guide RNAs with the distribution of cells expressing non-targeting control guide RNAs with the functional edit rate for a particular guide RNA. To determine an empirical FDR, we repeated this procedure for 1,000 iterations and calculated the rate at which cells expressing guide RNAs against a target were identified as more significantly distributed across regions compared to the random subset of non-targeting control cells. To obtain a score for the enrichment of differentially distributed targets (FDR < 0.1) across pseudospatial regions, we calculated the odds ratio for each target-region pair using fisher.test in R with the presence or absence of non-targeting control cells in the region and background as failures in our contingency tables. Targets accumulated in at least one region at an enrichment score (log, of the odds ratio) of 1 or higher were regarded as strongly enriched. Finally, hierarchical clustering of the enrichment score across pseudospatial regions for differentially distributed targets was used to visualize the accumulation of cells across pseudospace using the heatmap function in the pheatmap package specifying ward.D2 as the clustering method.

We assessed whether the distribution of guide RNA containing cells as well as a random subset of non-targeting control cells was significantly different compared to the larger pool of non-targeting control expressing cells across pseudospatial regions using a chi-square test. To determine an empirical FDR, we repeated this procedure for 1,000 iterations and calculated the rate at which cells expressing guide RNAs against a target were identified as more significantly distributed across regions compared to the random subset of non-targeting control cells. To obtain a score for the enrichment of differentially distributed targets (FDR < 0.1) across pseudospatial regions, we calculated the odds ratio for each target-region pair using fisher.test in R with the presence or absence of non-targeting control cells in the region and background as failures in our contingency tables. Targets accumulated in at least one region at an enrichment score (log, of the odds ratio) of 1 or higher were regarded as strongly enriched. Finally, hierarchical clustering of the enrichment score across pseudospatial regions for differentially distributed targets was used to visualize the accumulation of cells across pseudospace using the heatmap function in the pheatmap package specifying ward.D2 as the clustering method.

**Mean expression of enriched targets in HNSCC tumor cells.** Expressed cell surface receptors and transcription factors were identified as those expressed across a minimum of 50 cells in tested HNSCC tumors. To determine the mean expression levels of enriched targets from our CRISPR-Cas9 screen in HNSCC tumors we log10 normalized gene expression for the defined genes after library size normalization and addition of a pseudocount of 1. Then the mean expression level across all cells for a given tumor were averaged. Results were visualized as a heatmap of enriched target gene expression levels using the heatmap function in the pheatmap R package. Expression profiles were clustered specifying ward.D2 as the clustering method. A column annotation was added depicting the relative partial EMT rank for every tumor as observed by Puram et al.

**Statistical methods.** Differential gene expression analyses were performed using the differential gene test implemented in Monocle2 and test results corrected for multiple hypothesis testing using the Benjamini–Hochberg procedure. Wilcoxon rank-sum test was used to determine statistical significance of the differences in aggregate gene expression scores for cells across various treatments with correction for multiple hypothesis testing performed using the Holm procedure. Two-tailed Student’s t-tests were used to determine statistical significance of changes in EMT marker protein expression measured via flow cytometry.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
Data are available on GEO under accession number GSE114687. Data will also be provided via the Github repository described in ‘Code availability’.

**Code availability**
Code can be found on Github at https://github.com/cole-trapnell-lab/pseudospace.
Reporting Summary

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| ☑   | An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☑   | The statistical test(s) used AND whether they are one- or two-sided |
| ☑   | Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
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| ☑   | For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever suitable. |
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| ☑   | Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated |
| ☑   | Clearly defined error bars |
| ☑   | State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection: The single cells analysis pipeline cellranger (version 1.3.0, 10X Genomics) was used to collect the data used in this article.

Data analysis: The single cells analysis package Monocle2 (version 2.6.3) was used in this article. A copy of the analyses performed will be available for distribution on github upon publication.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
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Data is available on GEO under accession number GSE114687 and provided via a Github repository upon publication.
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | For the initial pseudospatial analysis experiments pertaining to Figures 1 and 2 we determined 2000 cells per fraction to be a reasonable number to robustly identify the underlying trajectory of cells in a well. For single-cell loss of function experiments (Figures 3 and 4), the number of cells for single cell RNA-Seq were determined by obtaining a reasonable amount of coverage in terms of minimum number of cells per target (roughly more than 50 cells per genotype). No statistical methods were used to predetermine sample sizes. |
| Data exclusions | No data exclusions |
| Replication | A quantitative comparison of the results of our initial pseudospatial experiments pertaining to Figures 1 and 2 to cells expressing non-targeting control guide RNAs within our pooled screen, pertaining to Figures 3 and 4, identified strong agreement between experiments. Flow cytometry experiments were performed in biological replicate (n = 4-7) and the mean and standard deviation from the mean of the measurements reported. Attempts at replication were successful. |
| Randomization | Not applicable |
| Blinding | Not applicable |

Reporting for specific materials, systems and methods

| Materials & experimental systems | Methods |
|---|---|
| n/a | n/a |
| Involved in the study | Involved in the study |
| Unique biological materials | √ ChIP-seq |
| Antibodies | √ Flow cytometry |
| Eukaryotic cell lines | √ MRI-based neuroimaging |
| Palaeontology | |
| Animals and other organisms | |
| Human research participants | |

Antibodies

Antibodies used

- Rabbit anti-E-cadherin, Cell Signaling Technologies, #3195 lot 13. Mouse anti-N-cadherin, Cell Signaling Technologies, #14215 lot 2. Rabbit anti-vimentin, Cell Signaling Technologies, #5741 lot 5. Mouse anti-cyttoplasmic-fibronectin, Abcam, #ab6328 lot GR31938080-1. Rat anti-CRB3, Abcam, #ab180835 lot GR32532558-1. Mouse anti-desmoplakin I+II, Abcam #ab16434 lot GR3232461-2. Mouse anti-pan-Keratin, Cell Signaling Technologies #4545 lot 1.

Validation

The specificity for all antibodies was confirmed by the manufacturer via immunoblotting confirming that antibodies recognize proteins at the expected molecular weights and via immunofluorescence staining confirming that antibodies recognize proteins with the expected sub-cellular localization. Additionally, anti-E-cadherin and anti-vimentin antibodies were validated for flow cytometry via comparison of e-cadherin low (Hela) and high (MCF7) cell lines and anti-vimentin antibody incubated cells vs. an IgG isotype control, respectively. All antibodies were validated by the manufacturer for specificity to their appropriate antigen.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

- MCF10A breast epithelium cells were purchased from ATCC (CRL-10317). Primary human mammary epithelial cells were purchased from Thermo-Fisher Scientific (A10565).
Authentication
MCF10A were not authenticated but used within 10 passages of purchase. All HuMEC experiments were performed with passage 4 cells.

Mycoplasma contamination
MCF10A cell were tested and confirmed negative for mycoplasma contamination.

Commonly misidentified lines (See ICLAC register)
Not a commonly misidentified cell line.

Flow Cytometry

Plots
Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
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- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
MCF10A and HuMEC cells were seeded in a cloning ring in the center of each well of a 6 well dish, the next day the ring was removed and cells allowed to undergo a spontaneous EMT with or without TGF-B. TGF-B was replenished every 48 hours. After 7 days, cells were harvested by trypsinization, washed twice with PBS, resuspended in 500 uL of cold PBS and 5 mL of ice cold ethanol were added drop-wise to cells while vortexing at low speed. Samples were washed twice with PBS containing 1% BSA (PBS-B) and blocked for 1 hour at room temperature wit PBS-B. Each sample was divided into two, a mix of rabbit anti-e-cadherin/mouse anti-fibronectin or rabbit anti-vimentin/mouse anti-n-cadherin added to one of the two aliquots and samples incubated for 2 hours at room temperature in PBS containing 1% BSA and 0.1% tryton X-100 (PBS-TB). After which, cells were washed twice with PBS-TB, and incubated for 1 hour at room temperature in a mix of Alexa-488 conjugated goat anti-mouse and Alexa-647 conjugated goat anti-rabbit secondary antibodies. Finally, cells were washed twice with PBS-TB and resuspended in PBS for flow cytometric analysis.

Instrument
Data was collected on a BD Bioscience LSRII.

Software
The data was collected using FACSDiva version 8 software. Data was analyzed using FlowJo 10.

Cell population abundance
All expected positive cell populations were present at an abundance of 15% or higher.

Gating strategy
Before analysis of fluorescence, single cells were isolated via sequential gating on SSC-A vs. FSC-A, FSC-H vs FSC-W and SSC-H vs SSC-W according to standard flow cytometry practices. Gates for APC-A (describing e-cadherin or vimentin levels) and FITC-A (describing n-cadherin or fibronectin) were set using the spontaneous EMT sample as a negative control for n-cadherin and fibronectin low populations and the TGF-B driven EMT as a negative control for e-cadherin and vimentin low populations.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.