A time- and matrix-dependent TGFBR3–JUND–KRT5 regulatory circuit in single breast epithelial cells and basal-like premalignancies

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Basal-like breast carcinoma is characterized by poor prognosis and high intratumour heterogeneity. In an immortalized basal-like breast epithelial cell line, we identified two anticorrelated gene-expression programs that arise among single extracellular matrix (ECM)-attached cells during organotypic three-dimensional culture. The first contains multiple TGF-β-related genes including TGFBR3, whereas the second contains JUND and the basal-like marker KRT5. TGFBR3 and JUND interconnect through four negative-feedback loops to form a circuit that exhibits spontaneous damped oscillations in three-dimensional culture. The TGFBR3–JUND circuit is conserved in some premalignant lesions that heterogeneously express KRT5. The circuit depends on ECM engagement, as detachment causes a rewiring that is triggered by RPS6 dephosphorylation and maintained by juxtacrine tenascin C, which is critical for intraductal colonization of basal-like breast cancer cells in vivo. Intratumour heterogeneity need not stem from partial differentiation and could instead reflect dynamic toggling of cells between expression states that are not cell autonomous.

Genetically identical cells often coexist in different molecular states1,2. Stochastic heterogeneities can drive cell fates in specific developmental contexts3,4. Within mature tissues, however, cell-autonomous heterogeneity is suppressed unless the molecular circuitry has been perturbed5. Cell-to-cell heterogeneity has been described in solid tumours, and heterogeneity within carcinoma cell lines has been associated with drug resistance6–8.

Heterogeneity cannot be entirely explained by random biological noise—there are substantial contributions from a cell’s local environment and its history9,10. For most epithelial tissues, tracking cell-to-cell variability in time and space is difficult11. Organotypic three-dimensional (3D) cultures allow monitoring of heterogeneity by supporting cells in reconstituted basement membrane12–14. The more realistic geometry and ECM context can give rise to non-genetic variations in molecular state15,16. For instance, ECM-adhesion receptors comprise most of the stem or progenitor markers for heterogeneity in breast tissue and breast cancer17,18. Organotypic heterogeneities might provide insight into clinical mechanisms of tissue and tumour heterogeneity that would otherwise be inaccessible.

Using 3D basement-membrane cultures of basal-like breast epithelia12,19, we have uncovered a dynamic heterogeneity that develops among ECM-attached cells during acinar formation. The expression circuit is composed of two anticorrelated transcriptional programs that establish a pair of expression states defined by TGFBR3 and JUND. When this circuit is spontaneously excited, ECM-attached cells oscillate transiently and asynchronously between states, creating the static appearance of a cellular mosaic. Single-cell TGFBR3–JUND regulation tracks with heterogeneity of a cytokeratin (KRT5) diagnostic for ductal carcinomas in situ with basal-like features (basal-like DCIS). KRT5 correlations reverse on detachment in vitro and in ECM-poor regions of basal-like DCIS, although availability of such samples was limited. We link the reversal to a keratinization process that is initiated by RPS6 dephosphorylation and maintained by expression of tenascin C (TNC). Disruption of TNC inhibits intraductal outgrowth of basal-like breast cancer cells in vivo, suggesting a functional role for the circuit during premalignancy.

The dynamic and ECM-dependent transition of individual tumour cells between expression states may relate to the poor prognosis of heterogeneous basal-like breast cancer20,21.
RESULTS

Two coexisting single-cell states defined by TGFBR3 and JUND

We recently described a random-sampling approach that profiles statistical fluctuations to uncover cell-to-cell heterogeneities in gene-expression regulation\(^{16,22}\). Applying this stochastic profiling technique to a basal-like MCF10A cell clone cultured in basement membrane (Supplementary Fig. 1) identified 547 transcripts subject to strong heterogeneous regulation. Seventeen per cent of transcripts fell into two clusters that were anticorrelated on a sampling-to-sampling basis (Fig. 1a,b). The first cluster included TGF-β receptor III (TGFBR3, a high-affinity TGF-β co-receptor\(^{23}\), growth differentiation factor 11 (GDF11, a TGF-β-family ligand\(^{24}\), and TGF-β-induced protein (TGFBI, an ECM molecule downstream of TGF-β-family signalling\(^{25}\)). The co-occurrence of a TGF-β receptor, ligand and marker protein suggested that the first cluster might be linked to TGFBR3-dependent signalling and gene expression.

Figure 1 TGFBR3 and JUND lie within anticorrelated single-cell expression programs among ECM-attached basal-like cells in organotypic 3D culture. (a) Hierarchical clustering of sampling fluctuations for the TGFBR3 and JUND anticorrelated expression programs identified by stochastic sampling of ECM-attached cells at day 10 of acinar morphogenesis\(^{16}\). 10-cell sampling data were scaled to log unit variance and clustered by Euclidean distance with Ward’s linkage. (b) Stochastic-profiling anticorrelations between JUND and TGFBR3, GDF11 and TGFBI. The Pearson correlation \((R)\) of \(n = 16\) independent 10-cell samples is indicated. (c) Three-colour RNA FISH images showing anticorrelated expression between JUND and TGFBI. Images are pseudocoloured to highlight quantitative differences in fluorescence intensity, and single cells showing strong anticorrelation are highlighted with arrows (high expression) or flat markers (low expression). A combination of three housekeeping genes (GAPDH, HINT1, PRDX6) was used as a control for total cellular mRNA levels\(^{42}\). (d) TGFBR3 and JUND show inverse frequencies of heterogeneous expression by RNA FISH. Active JUND and TGFBR3 transcription appears as nascent foci in the nucleus (arrows). Cells with weak expression are indicated with flat markers. Cells were counterstained with DAPI (blue) to label nuclei. (e) Quantification of TGFBR3 and JUND expression frequencies within matrix-attached cells. For c and d, scale bar is 20 \(\mu\)m. For e, data are shown as the mean \(\pm\) s.e.m. of \(n = 4\) independent hybridizations. For source data, see Supplementary Table 3.

The three TGF-β-related genes were strongly anticorrelated with the \(jun\) \(D\) proto-oncogene (JUND; Fig. 1a,b), the only transcription factor in the second cluster, which comprised mostly of protein biosynthetic genes\(^{16}\). We verified the single-cell anticorrelation by RNA fluorescence \(in\) \(situ\) hybridization (FISH) and showed that JUND and TGFBR3 were expressed at reciprocal frequencies in ECM-attached cells (Fig. 1c–e). TGFBR3 and JUND thus mark two states that basal-like cells spontaneously occupy when in contact with ECM.

TGFBR3–JUND heterogeneity is critical for normal acinar morphogenesis

TGFBR3 expression is strongly induced during organotypic culture\(^{26}\) (Fig. 2a). If TGFBR3 upregulation occurred sporadically, it could explain the heterogeneous expression pattern observed among single ECM-attached cells (Fig. 1d). To determine whether TGFBR3 induction was important for acinar morphogenesis, we depleted
Figure 2 TGFBR3 and JUND are functionally important for 3D morphogenesis. (a) Time-dependent expression of TGFBR3 during 3D culture. (b) Knockdown of TGFBR3 and inducible addback of murine RNAi-resistant Tgfbr3. TGFBR3/Tgfbr3 levels for cells cultured in the absence (lanes 1 and 2) or presence (lane 3) of 1 μg ml⁻¹ doxycycline for 24 h were analysed by immunoblotting. Hsp90 was used as a loading control. Densitometry of TGFBR3/Tgfbr3 abundance is shown normalized to the GFP shRNA control. (c,d) Blocking TGFBR3 induction specifically elicits a ductal-branching phenotype. The MCF10A-5E lines described in b were placed in morphogenesis in the absence (control) and TGFBR3 shRNA or presence (Tgfbr3 addback) of 1 μg ml⁻¹ doxycycline from day 4–10. Acini were fixed at day 10 of 3D culture, stained for E-cadherin (green) and HA-tagged Tgfbr3 (red), and analysed by confocal immunofluorescence. Cells were counterstained with DRAQ5 (blue) to label nuclei. (e) Constitutive expression of HA-tagged JUND analysed by immunoblotting. Densitometry of JUND abundance is shown normalized to pBabe vector control. (f,g) Constitutive JUND expression causes stable cribriform-like acinar structures. Acini from the MCF10A-5E lines described in e were placed in morphogenesis, fixed at day 28, stained for E-cadherin (green) and HA-tagged JUND (red), and analysed by confocal immunofluorescence. Cells were counterstained with DRAQ5 (blue) to label nuclei. (h) Homogenization of JUND expression by knockdown of TGFBR3 and addback with murine RNAi-resistant JunD to near-endogenous expression levels. JUND/JunD levels were determined by immunoblotting. Densitometry of JUND/JunD abundance is shown normalized to the GFP shRNA control. (i) Quantification of the cribriform-like phenotype at day 28 of 3D culture for the cells in h. For a,c,g,i, data are shown as the mean ± s.e.m. of n = 3 (a) or n = 4 (c,g,i) independent experiments. For d and f, scale bar is 20 μm. For e and h, tubulin was used as a loading control and *n.s. denotes a nonspecific band. For source data, see Supplementary Table 3. Uncropped images of blots are shown in Supplementary Fig. 9.

TGFB3 by RNA-mediated interference (RNAi) and verified specificity with a RNAi-resistant murine Tgfbr3 that is doxycycline-inducible (Tgfbr3 addback; Fig. 2b). Inhibiting TGFBR3 upregulation caused a profound ductal-branching phenotype in ~30% of acini expressing TGFBR3 short hairpin RNA (shRNA; Fig. 2c,d). Branching returned to baseline when Tgfbr3 was induced at day 4, the time when endogenous TGFBR3 levels normally begin to rise (Fig. 2a,c,d). Thus, TGFBR3 upregulation specifically suppresses ductal branching, conceivably by sensitizing cells to TGF-β-family ligands.

Unlike TGFBR3, JUND is easily detected under normal growth conditions and is frequently expressed in ECM-attached cells (Fig. 1e). To examine the role of sporadic JUND downregulation (Fig. 1d), we constitutively expressed HA-tagged JUND. This perturbation gave rise to stable cellular bridges across the acinar lumen, which are cytologically similar to the cribriform subtype of DCIS (ref. 27; Fig. 2e–g). Heterogeneous JUND downregulation remained critical until late in morphogenesis, because induction of HA–JunD at day 9 caused cribriform acini weeks later (Supplementary Fig. 2a,b). To exclude artefacts caused by mild JUND overexpression, we coexpressed a stable shRNA against JUND together with an RNAi-resistant murine JunD that restored near-endogenous levels (Fig. 2h). This homogenization of JUND expression also caused cribriform acini (Fig. 2i). Therefore, heterogeneous regulation of JUND is critically important for acinar morphogenesis of basal-like cells.

**TGFB3–JUND signalling is oscillatory and dynamically coupled**

To determine whether the TGFBR3–JUND clusters were functionally linked, we constitutively expressed TGFBR3 or JUND and analysed endogenous messenger RNA levels of the other cluster (Fig. 3a–c). Constitutive JUND expression downregulated both TGFBR3 (P = 0.0026, one-sided t-test; Fig. 3a) and TGFBI (P = 0.0027, one-sided t-test; Fig. 3b), suggesting that JUND antagonizes expression of the TGFBR3 cluster. Ectopic TGFBR3 expression reciprocally inhibited JUND expression (P = 0.022, one-sided t-test; Fig. 3c), indicating that JUND does not simply act as an upstream repressor of the TGFBR3 cluster. Mutual TGFBR3–JUND antagonism creates a double-negative (positive) feedback loop, which can establish two distinct molecular states.

Two other negative autoregulatory feedbacks were part of the overall wiring. Consistent with earlier reports, constitutive JUND...
Figure 3  JUND transcription and TGF-β-family signalling activity are functionally and dynamically coupled. (a,b) TGFBR3 and TGFBI are repressed by constitutive JUND expression. (c) Endogenous JUND is repressed by constitutive expression of TGFBR3 or JUND. (d) TGFBR3 is negatively regulated by TGF-β-family signalling. (e) Schematic of positive- and negative-feedback loops connecting TGFBR3 and JUND. The arrows and flat markers indicate the positive and negative relationships from a–d. Black circles indicate the two fluorescent reporters (RFP1–Smad2 and udsVenus (P
\( \text{uds}_\text{Venus} \)) used to monitor the single-cell dynamics of TGF-β-family activity and JUND promoter activity. (f,g) Multiple alignment of dynamic single-cell fluorescence trajectories. Two-colour live-cell confocal imaging was used to quantify the level of nuclear RFP1–Smad2 (f) and total udsVenus (P
\( \text{uds}_\text{Venus} \)) expression (g) of ECM-attached cells at day 10 of morphogenesis. Grey indicates no data. (h) Damped oscillations in an ordinary differential equation model of the TGFBR3–JUND expression circuit induced by TGFBR3 activation (left; RFP1–Smad2 range: [11.5–15.7], udsVenus (P
\( \text{uds}_\text{Venus} \)) range: [13.0–20.6]), TGFBR3 upregulation (middle; RFP1–Smad2 range: [12.1–23.2], udsVenus (P
\( \text{uds}_\text{Venus} \)) range: [0.745–18.4]), or JUND upregulation (right; RFP1–Smad2 range: [3.80–12.1], udsVenus (P
\( \text{uds}_\text{Venus} \)) range: [18.4–65.2]). In the model, the basal transcription rate was 4 h
\(^{-1}\), the basal translation rate was 100 mRNA
\(^{-1}\) h
\(^{-1}\), the mRNA degradation rate was 0.23 h
\(^{-1}\), the degradation of TGFBR3 protein was 3 h
\(^{-1}\), the degradation of JUND protein was 0.37 h
\(^{-1}\), the degradation of udsVenus was 2.8 h
\(^{-1}\), and the activation rate of TGFBR3 was 1 h
\(^{-1}\) (Supplementary Note 1). For a–c, MCF10A-5E cells stably expressing JUND–HA, TGFBR3–HA or vector control were placed in 3D culture and analysed at day 10 of morphogenesis by quantitative PCR for the indicated genes. Endogenous JUND was analysed with primers specific for the 3’ UTR of JUND. For d, MCF10A-5E cells were stimulated with 250 ng ml
\(^{-1}\) GDF11 for 4 h and analysed for TGFBR3 expression. Data are shown as the mean ± s.e.m. of n = 4 independent samples, and P values were calculated by Student’s one-sided t-test. For simulation code and source data, see Supplementary Data File 1 and Supplementary Table 3.

expression caused downregulation of endogenous JUND (P = 0.043, one-sided t-test; Fig. 3c), and TGFBR3 expression was acutely downregulated by TGF-β-family ligands (P = 1.4 × 10
\(^{-3}\), one-sided t-test; Fig. 3d). These findings delineate a hybrid signalling–transcriptional circuit comprised of one positive-feedback and two negative-feedback loops (Fig. 3e).

Regulatory circuits with interlinked positive and negative feedback can oscillate between molecular states\(^{26,31}\). We developed a live-cell imaging procedure for monitoring TGFBR3 and JUND activities simultaneously. Active TGF-β-family signalling (TGFBR3*) was tracked by RFP1-labelled Smad2 (Fig. 3e and Supplementary Fig. 3a,b). For JUND, we engineered a rapidly responsive fluorescent reporter of endogenous promoter activity (Fig. 3e). We inserted ~2 kilobases of the JUND promoter (P
\( \text{JUND} \)) upstream of the fast-maturing yellow fluorescent protein (YFP) variant, Venus\(^{52}\), which was destabilized by N-end rule fusion to ubiquitin C and carboxy-terminal fusion to a PEST sequence\(^{33,34}\) (Supplementary Fig. 3c–e). Coexpression of ultradestabilized Venus (udsVenus; P
\( \text{uds}_\text{Venus} \)) and RFP1–Smad2 did not substantially perturb acinar morphogenesis relative to control cultures (Supplementary Fig. 3f–h), suggesting that endogenous TGFBR3–JUND pathways were not markedly affected (Supplementary Fig. 3i–l). For 3D-culture experiments in which stable time-lapse imaging was successful, we repeatedly observed at least one ECM-attached cell with coupled dual-reporter dynamics (Supplementary Video 1).

To compile two-colour reporter activities across multiple experiments, we combined spectral filtering with algorithms from multiple-sequence alignment (Methods). The aggregate alignment revealed that both reporters exhibited transient peaks of activity separated by 5–10 h (Fig. 3f,g). When an ECM-attached cell remained in the optical plane long enough to observe two peaks, the second peak usually had a smaller amplitude than the first, suggesting pathway damping (Fig. 3f, upper rows; Fig. 3g, middle rows). When the two reporters were compared within the same cell, dynamics were antiphase at nearly all time points\(^{35}\). Asynchronous, antiphase dynamics within the TGFBR3–JUND circuit provide a mechanism for the static anticorrelation observed in fixed specimens (Fig. 1).

We next used computational modelling to determine whether the empirical circuit wiring could exhibit damped, antiphase responses such as those observed in live cells (Fig. 3e and Supplementary Note 1, and Supplementary Data File 1). The circuit was modelled as a system of ordinary differential equations containing JUND (mRNA and protein) and TGFBR3 (mRNA, protein and ligand-bound
protein—TGFBR3*). We assigned basal synthesis and degradation rates to mRNA and protein species, and used Hill functions to capture transcription and feedback (Methods and Supplementary Note 1). The live-cell reporters were encoded according to their mechanism of action and taken as outputs for the model.

We allowed the system to relax to steady state and then excited it with a modest 50% impulse of TGFBR3 activation, TGFBR3 transcription or JUND transcription. TGFBR3 activation yielded the clearest damped, antiphase oscillations (Fig. 3h), suggesting that the circuit could be endogenously triggered by TGF-β family ligands, such as GDF11 (Fig. 1b) and others, which reside and accumulate in the ECM (refs 36,37). We conclude that the specific feedback configuration is sufficient to cause the observed circuit dynamics (Fig. 3e–g).

The TGFBR3–JUND circuit is active in heterogeneous basal-like lesions

Cell-to-cell mosaicism is observed clinically in basal-like breast cancer, where ∼50% of cases are highly heterogeneous for subtype-diagnostic cytokeratins10,38. One such cytokeratin, KRT5, lies within the JUND clus...
To address this challenge, we collected an independent cohort of premalignant basal-like DCIS lesions with heterogeneous KRT5 expression. KRT5 indicates poor prognosis for basal-like carcinoma, and heterogeneous premalignancies would allow the cell-by-cell correlations of KRT5 to be examined with JUND and TGFB3 while the tissue architecture was still intact. We therefore focused on the 8% of hormone-negative specimens diagnosed as DCIS to avoid complications associated with invasion and metastasis. We identified 22 archival cases that met these criteria along with four normal tissues obtained by reduction mammoplasty (Supplementary Table 1). In normal breast tissue, KRT5 and TGFB3 were strongly expressed in the basal layer. KRT5 was predominantly localized to the ductal myoepithelia, whereas TGFB3 was expressed mostly in the lobular myoepithelia (Fig. 4b). Conversely, JUND protein was very low in normal tissue but increased substantially in basal-like DCIS, where TGFB3 was often undetectable (Fig. 4b). These results indicated a switch in TGFB3–JUND–KRT5 regulation during premalignancy.

Next, we examined the coexpression of KRT5 and TGFB3 or JUND in single cells by multicolour immunofluorescence. In the 59% of premalignant lesions where TGFB3 could be detected, TGFB3 and KRT5 expression remained mutually exclusive (Fig. 4c and Supplementary Fig. 4a–c). This single-cell anticorrelation was consistent with both our retrospective analysis of invasive carcinomas and stochastic profiling of basal-like cultures (Fig. 1a and 4a). Conversely, cases with KRT5-positive regions of primary DCIS (41% of total) exhibited a strong positive correlation between KRT5 and JUND among single cells (Fig. 4d and Supplementary Fig. 4d–f). Although the rarity of clinical samples limits the power of the present analysis, the agreement between the clinical and in vitro data suggests that basal-like ECM cultures might mimic the burst of proliferation and environmental stress experienced by early neoplasms.

**JUND–KRT5 coexpression is modulated by ECM context**

High-grade intraductal carcinomas frequently consist of a primary DCIS region along with secondary regions of clinging carcinoma. Clinging carcinoma forms when neoplastic cells disseminate intraluminally from the DCIS and canzerize peripheral breast lobules and ducts (Fig. 4e,f). Examination of clinging carcinoma regions showed anticorrelation of JUND and KRT5 (Fig. 4g and Supplementary Fig. 4g–i). JUND–KRT5 switching occurred without gross cytological changes in cases with both DCIS and clinging carcinoma (Fig. 4e,f,h–k). Tumour geography thus seemed to provide a form of external control on the TGFB3–JUND expression circuit and its co-regulation with KRT5.

The reversal of JUND–KRT5 coexpression prompted us to re-examine their relationship in vitro. During 3D culture, JUND and KRT5 proteins were coordinately expressed among outer cells (Fig. 5a). However, the JUND–KRT5 coexpression pattern was anticorrelated in interior cells. This transition could not have been anticipated by our initial profiling study, which focused exclusively on outer cells. Nonetheless, the finding provided an independent replication of the JUND–KRT5 switching observed in basal-like neoplasms (Fig. 4d,g). This observation was corroborated in a few exceptional cases of DCIS where cells had detached partly or entirely from the tumour margin and JUND–KRT5 coexpression was reversed (Supplementary Fig. 4j–l).

To identify the molecular basis for the JUND–KRT5 inversion, we considered the variegated microenvironments of ECM cultures and human tumours. The most-recognized difference within ECM cultures is the spatially segregated access to basement membrane and other ECM molecules basolaterally. Outer cells contact the ECM-rich culture support and secrete their own ECM molecules. Inner cells are deprived of both these ECM sources and thus should be starved for integrin engagement. Analogously, in regions of DCIS, the local tumour stroma is potently activated, providing ECM to the primary tumour. Cells in clinging carcinoma regions have left the primary site to colonize luminal, ECM-poor regions of the ductal tree and may behave like inner cells of the culture.

To simulate ECM deprivation, we placed cells in suspension culture. Before anoikis was evident, we observed clear and highly stereotyped changes in single-cell JUND–KRT5 expression. For the first 8 h, JUND–KRT5 were coexpressed as double-positive or double-negative cells (Fig. 5b,c). At 24 h, the JUND+–KRT5+ and JUND−–KRT5− subpopulations became more clearly separated, when KRT5 increased with a filamentous pattern (KRT53) and anticorrelations started to appear. By 48 h, JUND+–KRT55 cells had vanished, and a fourth keratinized state emerged with intense KRT5 staining and no JUND protein or nuclear DNA (KRT55; Fig. 5b,c). Live-cell imaging showed that progression to the JUND−–KRT58 state was rapidly executed, with keratinized skeletons eventually collapsing as cellular dust (Fig. 5c,d and Supplementary Fig. 5a). These late cellular steps are reminiscent of cornification, a cell-death process typically associated with skin.

Flow cytometry determined the high KRT5 (KRT5+) and KRT55 (KRT5−–DAPI−) cells to be minority populations (Fig. 5e,f and Supplementary Fig. 5b). Overall, KRT5 levels increased during suspension culture, agreeing with the very high expression of single KRT5+ cells (Fig. 5g and Supplementary Fig. 5b). KRT5 levels increased with two type I keratin partners (KRT14 and KRT15; Supplementary Fig. 5c), supporting the execution of a specific keratin program in JUND+–KRT55 cells. Vimentin remained mostly constant, but E-cadherin increased during keratinization and preceded the onset of anoikis by at least 24 h (Supplementary Fig. 5c).

Endogenous JUND levels increased transiently before KRT5 upregulation (Fig. 5g), suggesting a role in the sequelae of ECM detachment. Although ectopic expression of JunD left the induction of KRT5 protein unaltered (Supplementary Fig. 5d), JunD-over-expressing cells largely remained in a double-positive state without overt keratinization (P = 7.0 × 10⁻⁵ and 3.0 × 10⁻⁵), two-sided t-test; Fig. 5h,i). Conversely, JUND knockdown accelerated keratinization and augmented it, with KRT55 cells apparent as early as 8 h (P = 2.4 × 10⁻⁴ and 8.7 × 10⁻⁴, two-sided t-test; Fig. 5j,k), even though KRT5 upregulation was unaffected (Supplementary Fig. 5e). We conclude that JUND restrains detachment-induced keratinization but is independent of the upregulation of KRT5 itself.

**Detachment-induced KRT5 upregulation is triggered post-transcriptionally by loss of phosphorylated RPS6**

To identify the mechanism of detachment-induced KRT5 upregulation, we used 3D ECM cultures and small-sample...
Figure 5 JUND and KRT5 become anticorrelated through keratinization induced by prolonged ECM detachment. (a) The JUND-KRT5 correlation state depends on attachment to basement membrane during epithelial acinar morphogenesis. MCF10A-5E acini were fixed at day 10 of morphogenesis, stained for KRT5 (green) and JUND (red), and analysed by confocal immunofluorescence. Cells were counterstained with DRAQ5 (blue) to label nuclei. (b,c) Loss of ECM-attachment induces keratinization and JUND-KRT5 anticorrelation. MCF10A-5E cells were placed in polyHEMA-coated plates with assay medium containing 5 ng ml\(^{-1}\) EGF. (d) Epithelial keratinization occurs rapidly during cell detachment. MCF10A-5E cells stably expressing JUND-Venus (false-colour red) and RFP1–KRT5 (false-colour green) were placed in suspension with assay medium containing 5 ng ml\(^{-1}\) EGF. Two-colour live-cell confocal images were collected after the indicated times in suspension. (e,f) Flow cytometry quantification of high KRT5 (KRT5\(^{+/+}\)) and keratinized (KRT5\(^{-/-}\))-DAPI\(^{-/-}\) cells as a function of time in suspension. (g) KRT5 protein expression is upregulated during detachment. MCF10A-5E cells were placed in suspension for the indicated times.

(h–k) JUND delays the terminal steps of keratinization. MCF10A-5E cells were transduced with doxycycline (DOX)-inducible JUND-HA (h,i) or JUND shRNA or GFP shRNA control (j,k) and placed in suspension culture for the indicated times. For a and b, correlated and anticorrelated regions of expression are indicated with arrows and rectangles respectively. For b,c,i, and k, cells were fixed at the indicated times, stained for KRT5 (green) and JUND (red), and analysed by confocal immunofluorescence. Cells were counterstained with DAPI (blue) to label nuclei. For g, JUND and KRT5 levels were analysed by immunoblotting with Hsp90, tubulin and actin used as loading controls. JUND appears as both short (JUND\(_{S}\)) and long (JUND\(_{L}\)) forms. For e,f,h, and j, flow cytometry data are shown as the mean ± s.e.m. of \(n=4\) independent biological samples, and \(P\) values were calculated by Welch’s two-sided \(t\)-test. For a,b,d,i and k, scale bar is 20 \(\mu\)m. For c, scale bar is 10 \(\mu\)m. For e,i and k, single cells representing intermediate stages of keratinization are highlighted with arrows. For source data, see Supplementary Table 3. Uncropped images of blots are shown in Supplementary Fig. 9.

Complementary DNA amplification\(^{16,22}\) to profile mRNA expression globally within developing acini (Fig. 6a). Matrix-attached and matrix-deprived cells were isolated by laser-capture microdissection at day 6, when inner versus outer cell fates have just stabilized but later programs have not yet been fully engaged\(^{15,42}\). Transcriptional profiling of each subpopulation revealed divergent regulation of JUND and KRT5 mRNA. KRT5 levels were proportionally lower (and JUND levels higher) in inner ECM-deprived cells, contrasting the changes in KRT5-JUND protein observed in suspension (Figs 5g and 6b). To exclude that the discrepancies were due to differences in assay format, we quantified KRT5 and JUND transcripts in suspension and observed similar mRNA changes as in microdissected cells (\(P = 0.014\) and
1.5 × 10^{-4}, two-sided t-test; Fig. 6c,d). This indicated that KRT5 protein upregulation did not result from increased gene expression.

Surveying the entire cluster for matrix-dependent transcripts that correlated with KRT5 or JUND, we identified the ribosomal protein RPS6, which showed proportionally decreased expression in ECM-deprived inner cells (Fig. 6e). Unlike KRT5, RPS6 was not downregulated in suspension cells (Fig. 6f). We attribute this difference to the RPS6 increase that occurs with time in 3D culture, presumably in matrix-attached cells (Fig. 6g). Notwithstanding, we found that RPS6 was required for detachment-induced upregulation of KRT5, with keratinization observed only in cells that had escaped knockdown (Supplementary Fig. 6a-d). This implicated a translational mechanism that depended specifically on the status of RPS6.

RPS6 is the canonical substrate of S6 kinase (S6K), which is activated by integrin engagement and inactivated by detachment^{49,50}. When 3D cultures were stained for RPS6 phosphorylation (p-RPS6), we observed no immunoreactivity among inner cells (Fig. 6h). In suspension culture, loss of p-RPS6 occurred acutely after 8 h (Fig. 6i). To reconstitute p-RPS6, we inducibly overexpressed a constitutively active S6K (E389-ΔCT) mutant^{51} (Supplementary Fig. 6e,f). Doxycycline treatment at 8 h after detachment maintained a suprabasal level of p-RPS6 in a fraction of the population and attenuated keratinization at 24 h (P = 0.014, two-sided t-test; Fig. 6j–l and Supplementary Fig. 6g). To determine whether RPS6 dephosphorylation was sufficient to induce keratinization, we treated adherent cells with inhibitors of TORC1 or S6K (Supplementary Fig. 6h). All inhibitors reduced RPS6 phosphorylation and caused sporadic keratinization in attached cells, whereas MEK1/2 inhibition did not (Supplementary Fig. 6i). Thus, loss of p-RPS6 is a critical trigger for detachment-induced keratinization of basal-like breast epithelial cells.

Stabilization of anticorrelated JUND–KRT5 by TNC

Keratinization provided an appealing mechanism for the JUND–KRT5 anticorrelation observed in inner cells and clinging carcinoma regions of basal-like breast cancer (Figs 4g and 5a). However, it was unclear why JUND^{ΔCT}–KRT5^{ΔP} cells would exist if detachment from ECM rapidly caused irreversible loss of JUND and upregulation of KRT5. The JUND–KRT5 staining pattern revealed that keratinized JUND^{ΔCT}–KRT5^{ΔP} cells were often surrounded by JUND^{ΔCT}–KRT5 cells (Fig. 5a), suggesting that JUND^{ΔCT}–KRT5^{ΔP} cells could be exchanging juxtacrine signals with JUND^{ΔCT}–KRT5 cells.

To identify candidate ECM ligands that could fulfil this role, we screened breast cancer immunohistochemistry images from The Human Protein Atlas^{52,53}. Among 71 ligands^{54}, only the matricellular protein TNC (ref. 55) was expressed heterogeneously in a cell-intrinsic manner within clinging carcinoma regions of breast carcinoma...
Figure 7  JUND–KRT5 mosaicism in ECM-poor microenvironments is stabilized by TNC. (a,b) The JUND–KRT5 anticorrelation state reflects a microenvironment that lacks basement membrane but contains TNC. Day-10 frozen sections of MCF10-5E acini (a) and paraffin sections from premalignant basal-like neoplasms (b) were stained for KRT5 (green), JUND (red) and TNC (white) and imaged by wide-field immunofluorescence. (c) TNC protein expression is upregulated during detachment. MCF10A-5E cells were placed in suspension for the indicated times. Cells were fixed and stained for KRT5 (green), JUND (red) and TNC (white) and analysed by confocal immunofluorescence. Cells were counterstained with DAPI (blue) to label nuclei. (d) The JUND–KRT5 correlation is reversed in vitro by exogenous TNC. MCF10A-5E cells were grown on coverslips in assay medium +5 ng ml⁻¹ EGF in the presence or absence of 5 μg ml⁻¹ TNC for 8 days. The cells were stained with antibodies against KRT5 (green) and JUND (red) and imaged by wide-field immunofluorescence. Nuclei were counterstained with DAPI (blue). In the first two panels, single-colour fluorescence images are pseudocoloured to highlight quantitative differences in immunoreactivity. Dashed lines separate regions that stain strongly or weakly for KRT5 expression. (e,f) An agent-based model requires a TNC-like molecule to stabilize JUND–KRT5 expression patterns. Solid lines highlight clusters of locally homogeneous JUND expression (red). (g) Paraffin sections from early basal-like carcinomas were stained for KRT5 (green) and JUND (red) and imaged by wide-field immunofluorescence. Nuclei were counterstained with DAPI (blue). In the first two panels, single-colour fluorescence images are pseudocoloured to highlight quantitative differences in immunoreactivity. Strings of keratinized cells (solid) and clusters of local JUND homogeneity (dotted) are highlighted. For a–d and g, scale bar is 20μm. For simulation code, see Supplementary Data File 2.

(Supplementary Table 2 and Supplementary Fig. 7a,b). TNC is important in early colonization of breast-cancer metastases to the lung. Sporadic TNC expression has also been noted in basal keratinocytes, suggesting a connection to epidermal keratinization. We reasoned that TNC could stabilize JUND⁺–KRT5⁻ cells if it were endogenously expressed in vitro and in vivo.

In ECM cultures, we found inner cells that strongly expressed TNC (Fig. 7a). Interestingly, JUND⁺–KRT5⁻ cells seemed to extend lamellipodia around TNC⁺–JUND⁻–KRT5⁻ skeletons (Fig. 7a, inset), suggesting extensive adhesive contacts. In clinging carcinoma regions of basal-like premalignancies, TNC⁺–JUND⁻–KRT5⁺ cells were similarly in direct apposition with cells that were JUND⁺–KRT5⁻ (Fig. 7b and Supplementary Fig. 7c–h). Following ECM withdrawal, TNC was strongly upregulated in the KRT5⁵ and KRT5⁷ subpopulations (Fig. 7c and Supplementary Fig. 7i–k). Unlike other keratinization-related programs, TNC upregulation may be transcriptionally mediated (Supplementary Fig. 7j). Only 2–6% of detached cells expressed TNC, but ~60% of keratinized cells were TNC⁺ (Supplementary Fig. 7l–n). When TNC was added to 2D cultures of basal-like breast epithelia, the single-cell JUND–KRT5 correlation reversed (Fig. 7d), illustrating that TNC actively participates in anticorrelating JUND and KRT5 expression.

To determine whether TNC could explain the JUND–KRT5 mosaicism in ECM-poor microenvironments, we built a multi-cell agent-based model of clinging carcinoma (Supplementary Note 1 and Supplementary Data File 2). We coded for an arbitrary clinging carcinoma geometry, where individual cancer cells (agents) spontaneously keratinize as a function of their JUND–KRT5 levels and the neighboring expression of TNC (see Methods). Without TNC, virtually all cells keratinized (Fig. 7e), consistent with the irreversibility of keratinization in the model. In contrast, including TNC caused a stable mosaic of cells that were JUND⁻–KRT5⁵ or JUND⁺–KRT5⁻ (Fig. 7f). This model made two predictions that were subsequently verified in clinical specimens. First, keratinization should be extensive among cells immediately adjacent to the lumen because of fewer opportunities to be stabilized by adjacent TNC-positive cells (Fig. 7f, solid). Retrospectively, we identified many stretches of keratinized cells along clinging carcinoma lumina (Fig. 4g and 7g).
and Supplementary Figs 4g,i and 7c,g). Second, the model predicted multi-cellular clusters that were locally homogeneous for JUND (Fig. 7f, dashed), because JUND increases up until keratinization occurs (Fig. 5g) and TNC-positive cells corral the multi-cellular clusters at different times during the model simulation. A similar mechanism may operate in clinging carcinoma, because we observed several multi-cell clusters with roughly equal JUND expression, even though lesions were heterogeneous overall (Fig. 4g and 7g and Supplementary Figs 4g–i and 7c,g). We conclude that keratinization—triggered by detachment-induced RPS6 dephosphorylation and modulated by TNC—is responsible for the single-cell anticorrelation of JUND–KRT5 in basal-like clinging carcinoma.

**TNC promotes intraductal colonization of detached basal-like breast cancer cells in vivo**

We sought to examine the importance of keratinization for cancer cell survival in realistic ECM-poor environments. Following prolonged detachment, the basal-like breast cancer line MDA-MB-468 showed reduced RPS6 phosphorylation and JUND expression and upregulation of KRT5 (Fig. 8a). Pharmacologic inhibition of RPS6 also caused keratinization in attached MDA-MB-468 cells (Supplementary Fig. 8a), indicating that the JUND–KRT5 circuitry was largely preserved in this cancer line. Similar results were obtained with a variant of MCF10A cells expressing oncogenic Ras (MCF10DCIS.com59; Supplementary Fig. 8b,c), illustrating that the circuit is not disrupted by cell transformation. When MDA-MB-468 cells were injected intraductally into SCID-beige mice60, they resembled detached neoplastic cells in basal-like premalignancies (Fig. 8b,c).

To evaluate the importance of TNC in ECM-limited micro-environments in vivo, we transduced luciferase-expressing MDA-MB-468 cells with an inducible shRNA targeting TNC (ref. 56; Fig. 8d) and monitored tumour-cell survival following intraductal injection. TNC knockdown significantly increased the percentage of KRT5+ cells (P = 0.0014, two-sided t-test; Figs 7e and 8e). Two days after injection, we observed comparable bioluminescence in ducts injected with TNC knockdown cells compared to uninduced controls (Supplementary Fig. 8d). At 21 days, however, colonization frequency was significantly inhibited by TNC knockdown (P = 0.049, Fisher’s exact test; Fig. 8f).

When TNC shRNA cells were cultured in suspension, the pattern of TNC immunohistochemistry was similar to that observed in organotypic 3D cultures, with TNC-positive cells staining the ductal basement membrane and the tumour stroma (Fig. 8g). JUND and KRT5, which are co-regulated in attached MDA-MB-468 cells, were also co-upregulated in detached MDA-MB-468 cells with TNC knockdown (Fig. 8h). These results suggest that TNC promotes intraductal colonization of basal-like breast cancer cells in vivo.
of apoptotic cells was also significantly affected \( (P = 0.0002, \text{two-way analysis of variance}; \text{Supplementary Fig. 8e}). \) Analysis of injected glands with detectable bioluminescence showed viable proliferating cells together with evidence of anoikis (Supplementary Fig. 8f). There was strong TNC immunoreactivity next to viable cells in control ducts, which was virtually eliminated in ducts injected with induced TNC shRNA cells (Fig. 8g,h). Among the largest tumours, TNC expression exhibited a punctate pattern analogous to that observed in human breast cancers (Fig. 8i). Human TNC expression was also detected in the largest doxycycline-treated tumour, indicating that it had escaped TNC knockdown (Supplementary Fig. 8g). These xenograft experiments suggest that TNC provides a critical survival signal for neoplastic cells that would otherwise undergo keratinization or anoikis during premalignancy. Our results indicate that the TGFBR3–JUND–KRT5 expression circuit exhibits its own internal dynamics but is also subject to external control by the local ECM in vitro and in vivo.

**DISCUSSION**

By profiling expression heterogeneities in a relevant ECM context, we have uncovered a major signalling circuit within basal-like breast epithelia. Cells in contact with basement membrane undergo transient oscillations between two molecular states defined by their TGFBR3–JUND expression. Perturbation of either state disrupts normal acinar morphogenesis. Proper dynamic regulation of the circuit is critical for establishing and stabilizing the identity of ECM-attached cells. By extension, proliferating neoplasias may re-engage the TGFBR3–JUND circuit in search of a cell fate amidst a heterogeneous ECM microenvironment.

Our study began with a transcriptional dichotomy between two single-cell expression states, but the overall circuit extends beyond transcription. Circuit activation in ECM-attached cells probably occurs by post-translational signalling from TGF-β-family receptors. Interestingly, TGF-β ligands bind ECM and exist as latent complexes that become disinhibited by mechanical force61. Considering that breast epithelia are known to be mechanoresponsive62, the earliest trigger for circuit oscillations may be changes in local cell–ECM mechanics.

There also seems to be a critical post-transcriptional component regulated by phosphorylation of RPS6. Phosphorylated RPS6 often promotes selective translation of certain mRNA species, but p-RPS6 and translation can be uncoupled63. Surprisingly, KRT5 levels increase, rather than decrease, following loss of RPS6 phosphorylation. This could be due to increased KRT5 translation or improved stability as a filament pair with a type I keratin. Other type II keratins are post-transcriptionally regulated64, although the precise mechanisms remain elusive.

The ECM-dependent relationship between KRT5 and TGFBR3–JUND is reflected both in basal-like cultures and preinvasive basal-like neoplasms (Fig. 8j). Outer ECM-attached cells of cultured acini and primary DCIS show correlated expression of JUND–KRT5. In contrast, the inner ECM-deprived cells of a 3D acinus may mimic facets of preinvasive dissemination that partly explain the macroscopic heterogeneity of clinical specimens. Detached epithelial cells stochastically execute a keratinization program65, which delays anoikis by creating a TNC mosaic within 3D cultures and in clingo carcinoma. Breast-cancer patients with TNC-positive tumour cells frequently have lymph-node metastases and very poor prognosis66. Our data build on recent animal models66 by suggesting that juxtacrine TNC may be critical for secondary orthotopic colonization within the duct.

The most-recognized driver of late-stage tumour heterogeneity is genomic instability, but how heterogeneous tumours evolve from premalignancy has been more enigmatic. Our work places renewed emphasis on the microenvironment and the dynamic synchronicity of the constituent cells. Reversible lineage switching has been described in several contexts67,68, suggesting together with our results that breast cancer may be more dynamic than previously appreciated.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGEMENTS**

The authors thank R. Horwitz and I. Macara for critically reading an early version of this manuscript, the UVA Women’s Oncology Group for guidance, I. Fraser for plasmid reagents, L. Wang for help with the quantitative PCR experiments, and P. Pramoojnao for help with processing the clinical samples. This work was supported by the American Cancer Society (120668-RSG-11-047-01-DMC to K.A.J.) and the National Institutes of Health Director’s New Innovator Award Program (1-DP2-OD006640 to K.A.J.). K.A.J. is further supported by the Pew Scholars Program in the Biomedical Sciences and the David and Lucile Packard Foundation. C.C.W. is supported by a Breast Cancer Research Program Postdoctoral Fellowship Award from the Department of Defense (W81XWH-11-1-0037). S.S.B. is supported by a Graduate Research Fellowship from the National Science Foundation. L.J. is supported by a Harrison Fellowship from the University of Virginia.

**AUTHOR CONTRIBUTIONS**

C.C.W. performed all 3D and suspension experiments, acquired all live-cell and confocal images, cloned the cDNA and reporter constructs, and together with S.S.B. performed the in vivo experiments. S.S.B. designed the multiple-alignment algorithm, built the TGFBR-JUND circuit model and the agent-based model of clingo carcinoma, and together with C.C.W. performed the in vivo experiments. L.J. performed all immunofluorescence and wide-field imaging of clinical and suspension samples and completed the retrospective analysis of microarray data. K.A.A. acquired the clinical specimens and supervised the histological analysis. K.A.J. performed the small-sample cDNA amplification and supervised the project. All authors contributed to the design and interpretation of experiments. C.C.W. and K.A.J. wrote the manuscript with edits from L.J., S.S.B. and K.A.A.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Published online at www.nature.com/doifinder/10.1038/ncb2930

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METHODOLOGY

Cell lines and 3D culture. The MCF10A-5E clone was previously reported and was grown in organotypic 3D culture as described for MCF10A cells.21,22 MCF10DCIS.com cells were originally obtained from Wayne State University and cultured in L-15 medium (In Vitrogen) plus 10% fetal bovine serum (HyClone) without supplemental CO2.

Plasmids. pLKO.1 green fluorescent protein (GFP) shRNA puro (Addgene number 12273), pLKO.1 TGFBR3 shRNA puro (TRCN0000033430) and pLKO.1 JUND shRNA puro (TRCN0000014974) were obtained from The RNAi Consortium or Addgene. pBabe JunD-HE neo, pBabe JUND-HE neo, pBabe RFP1-Smad2 neo, pBabe JUND-Venus puro and pBabe RFP1-KRT5 hygro were constructed by PCR cloning from plasmid templates (Open Biosystems) into the retroviral vector pBabe neo, pBabe puro or pBabe hygro. 5′E6 (E389-ΔCT) was excised from pER7-HA-S6K1-E389-ΔCT (ref 3) by restriction digest with XbaI and EcoRI. Doxycycline-inducible Tgbr3-ba, Jund-ΔHA neo and 5′E6 (E389-ΔCT) expression vectors were constructed by PCR cloning or subcloning into the entry vector pENT_TrimRC2, followed by LR recombination into the lentiviral vector pSLIK neo.23 The human TRIPZ lentiviral-inducible RP6 shRNA constructs VTHS_333416 (number 1) and VTHS_333414 (number 2) and the TNC shRNA construct V2THS_133229 (ref 56) were obtained from Open Biosystems. pLenti PGK Blast V5-LUC (w528-1) was obtained from Addgene.

pTRF1 udsVenus (PmTRF) was constructed starting with the commercial lentiviral vector, pTRF1-mCMV-dsGFP (System Biosciences). First, coral GFP (gfp) was excised from pTRF1-mCMV-dsGFP by restriction digest with HindIII and EcoRV, and the vector was ligated with a similarly digested Venus24,25 prepared by PCR cloning designed to contain the appropriate motif for N-endo rule degradation25,26. The resulting pTRF1-mCMV-dsVenus was then digested with HindIII, dephosphorylated and ligated with a similarly digested ubiquitin C monomer prepared by PCR cloning from oligo(dT)-primed MCF10A-5E cDNA to produce pTRF1-mCMV-udsVenus. Last, this vector was digested with EcoRI and Spel and ligated with a similarly digested PmTRF prepared by PCR from MCF10A-5E genomic DNA to produce pTRF1 udsVenus (PmTRF). All constructs were verified by sequencing.

Transfection. Lentiviruses were prepared in HEK293T cell cultures (ATCC) by triple transfection of the lentiviral vector together with pSPAX2 and pMD.2G (Addgene) and transduced into MCF10A-5E, MDA-MB-468 and MCF10DCIS.com cells as described previously27. Retrosiruses were similarly prepared by double transfection of the pBabe construct together with pCL amphi (Addgene) and transduced into MCF10A-5E, MDA-MB-468 and MCF10DCIS.com cells as described previously28. Retroviruses were similarly prepared by double transfection of the pBabe construct together with pCL amphi (Addgene) and transduced into MCF10A-5E cells as described previously29. For viral vectors carrying selectable markers, transduced cells were selected in growth medium containing 2 μg ml−1 puromycin, 300 μg ml−1 G418, 100 μg ml−1 hygromycin or 4–6 μg ml−1 blasticidin until control plates had cleared. For addback experiments, viral titres were adjusted to match the endogenous protein expression as closely as possible. For live-cell reporters, we used the minimum viral titre that gave sufficient signal above the endogenous protein expression as closely as possible. For live-cell experiments, we used the minimum viral titre that gave sufficient signal above background for long-term imaging. For pTRF1 udsVenus (PmTRF), which lacks a selectable marker, transduced cells were flow sorted for baseline Venus fluorescence at the University of Virginia Flow Cytometry Core Facility.

RNA FISH. Acinar cultures were embedded at day 10 of morphogenesis. Single- and multi-colour RNA FISH on 5 μm cryosections was performed as described previously30,31. Quantitative PCR. Quantitative PCR was performed as described elsewhere32. Primer sequences are as follows: TGFBR3, 5′-tgacagcggccagcatttc-3′ (forward), 5′-acagcggttcatcatgta-3′ (reverse); JUNDF, 5′-ctcgccacccgggaa-3′ (forward), 5′-ctcctggcagctgtgatgttct-3′ (reverse); JUND, 5′-ctcgccacccgggaa-3′ (forward), 5′-ggtcagcgaagccgtaa-3′ (reverse); KR7, 5′-ttgctccatcactcctc-3′ (forward), 5′-ctctggattaaacagggc-3′ (reverse); RPS6, 5′-cctgccttaaagctgtggtaa-3′ (forward), 5′-ctcgccacccgggaa-3′ (reverse); TNC, 5′-ccacccaggggaacttggtaa-3′ (reverse); TGGC, 5′-ctctggattaaacagggc-3′ (forward).

Immunoblotting. MCF10A-5E cells expressing the indicated constructs were lysed in 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate. Odyssey extract (20–30 μg) was separated on a 8% or 10% SDS–PAGE gel and transferred to PVDF (Immobilon-FL, Millipore). Membranes were blocked with 0.5% Odyssey blocking buffer (LI-COR, 1:1 in PBS) and incubated overnight at 4°C in 0.5× Odyssey blocking buffer (LI-COR) or 0.1% Tween, then washed 4× in PBS at room temperature for 10 min in PBS and incubated for 1 h at room temperature in secondary antibody solution (0.5× Odyssey blocking buffer + 0.1% SDS + 0.1% Tween) containing IRdye 800- or IRdye 680LT-conjugated secondary antibody (1:2000, LI-COR). Membranes were washed 4× in PBS at room temperature and imaged by infrared fluorescence on a LI-COR Odyssey instrument. Relative band intensities were quantified by densitometry with ImageJ.

Clinical samples. The pathology database at the University of Virginia from 2004–2012 was searched for all high-grade DCIS, because this is the cohort that contains the basaloid subgroup. The set was then subclassed for ER status and only those that were ER negative were selected. The search was confined to 2004 and later because 2004 was the year pathologists began reflexively testing DCIS for ER status. All cases with an invasive carcinoma component were excluded. This resulted in 5–7 cases per year. The cases were deidentified for any patient demographics and used for immunohistochemical analysis of cytokeratin 5/6. Samples that were positive for cytokeratin 5/6 were followed up with a panel of six immunohistochemical markers: p16 (1/22 positive), E-cadherin (22/22 positive), RNF43 (22/22 positive), SMAD2 (22/22 positive), SMAD4 (22/22 positive), smooth-muscle actin (22/22 positive) and vimentin (6/22 positive; Supplementary Table 1). All clinical work was done according to a protocol under IRB-HSR approval number 14176 and PRC approval number 1363 (02-99).

Immunofluorescence. Immunofluorescence in frozen sections or on coverslips was performed as described previously33 using the following primary antibodies: cytokeratin 5/6 (DS/16 B4; 1:200, Dako, M2737), keratin 5 (1:5,000, Covance, SIG-3475), keratin 8 (BC-2; 1:2,000, Sigma, T2551), Jun D (329; 1:500, Santa Cruz, sc-7497), and phospho-S6 ribosomal protein (Ser 240/244; D6888; 1:1,000, Cell Signaling, number 5364), α-β-tubulin (1:1,000, Cell Signaling, number 2148), α-tubulin (1:20,000, Abcam, ab89984), β-actin (1:1,000, Ambion, number AM4302), keratin 5 (1:1,000, Covance, SIG-3475), keratin 14 (1:1,000, Covance, PRB-155P), Smad2 (L16D3; 1:1,000, Cell Signaling, number 3103), GFP (1:1,000, Invitrogen) and incubated for 1 h at room temperature in secondary antibody solution (0.5× Odyssey blocking buffer + 0.1% SDS + 0.1% Tween) containing IRdye 800- or IRdye 680LT-conjugated secondary antibody (1:2000, LI-COR). Membranes were washed 4× in PBS at room temperature and imaged by infrared fluorescence on a LI-COR Odyssey instrument. Relative band intensities were quantified by densitometry with ImageJ.

DOI: 10.1038/ncb2930

METHODS

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staining intensity. A minimum of 150 cells was scored per hybridization across four independent hybridizations.

**Time course alignment.** Data from live-cell imaging time courses were spectrally decomposed and aligned in the Fourier domain by low-pass filtering at 8.6 × 10^{-5} Hz, and then reconstructed with the ifft function in MATLAB. After spectral filtering, individual time courses were standardized and clustered hierarchically on the basis of the joint alignment of the RFP1–Smad2 and udsVenus traces between experiments. The alignment algorithm is based on a full sliding window of both traces with zero gaps and a cost function that uses the sum-of-squared difference between the two experiments to be aligned, scaled by the extent of overlap between them. All possible experiment pairs and alignments within the data set were considered, and the experiments with the best pairwise alignment were combined by average linkage. The exhaustive pairwise comparisons and linkages were repeated until all of the independent experiments were aligned. In the final pairwise comparison, one of two nearly equivalent alignments was visually selected.

**Computational modelling.** The TGFBR3–JUND circuit was modelled as a system of coupled ordinary differential equations. mRNA and protein species were assigned basal synthesis and degradation rates as described in Supplementary Note 1. Transcriptional inhibition steps were modelled using the Hill equation without cooperativity, and feedback strengths were adjusted manually as free parameters to reproduce the damped periodicity observed experimentally. Sensitivity analysis on the manually adjusted parameters is described in Supplementary Note 1. The model was simulated with ode15s in MATLAB and allowed to reach steady state before exciting the system with a 50% increase in the appropriate reaction rate for 1 h. Code for the simulations in Fig. 3h is available in Supplementary Data File 1.

The agent-based model of clonogenic carcinoma was constructed using NetLogo v4.1.1 (http://ccl.northwestern.edu/netlogo/). Single cells were seeded at a predefined geometry and initialized with the same basal level of KRT5 and JUND. During each time step of the simulation, KRT5 and JUND were incremented by a uniform pseudorandom number between zero and one [U(0,1)] that was inversely scaled by the number of neighboring TNC-positive cells (TNC−) as follows: (U(0,1))/(2^{TNC}). As JUND inhibits late keratinization (Fig. 5i), the fluctuating JUND–KRT5 difference was used as a proxy for keratinization. The JUND–KRT5 difference was evaluated after each time step, and keratinization occurred when the difference reached a critical negative threshold. Keratinized cells then expressed TNC and were no longer incremented for KRT5 or JUND expression. The model was run until steady state, and the final display was used as the model output. Simulations with alternative geometries are described in Supplementary Note 1, and code for the models in Fig. 7e,f is available in Supplementary Data File 2.

**Clustering analysis.** All unsupervised hierarchical clustering analysis was performed in MATLAB (Mathworks) using the Bioinformatics toolbox with Euclidean distance and Ward’s linkage. For the retrospective analysis of clinical samples, the microarray data set was mined for probe sets matching the genes in the TGFBR3 and JUND clusters (Fig. 1a). Probe sets were median centred and scaled to the interquartile range before clustering; TGFBR3 and JUND enrichment within the final dendrogram was assessed by a hypergeometric test.

**Immunohistochemistry.** Paraffin tissue sections (4 μm) were processed according to the optimal conditions for the target antigen. For TGFBR3 and KRT5, antigen retrieval and deparaffinization were performed with a PT Link (Dako) using low-pH (KRT5) or high-pH (TGFBR3) EnVision FLEX Target Retrieval Solution (Dako) for 20 min at 97°C. For JUND, sections were dewaxed through a graded alcohol series and used without antigen retrieval. Endogenous peroxidases were blocked with peroxidase and Alkaline Phosphatase Blocking Reagent (Dako) according to the manufacturer's recommendations. Sections were then incubated with one of the following primary antibodies for 30 min at room temperature: TGFBR3 (1:100, Sigma, HPA008257), cytokeratin 5/6 (D5/6B 4; 1:100, Dako, M7237), keratin 5 (1:5,000, Covance, SIG-3475), tenasin (BC-24; 1:2,000, Sigma, T2551), Jun D (329; 1:200, Santa Cruz, sc-74), ER (1:100, Biocare Medical, ACA 301), p53 (1:200, Dako, M7001), E-cadherin (1:1,000, Epitomics, 1702-1), KRT18 (1:100, epitope 1433-1), p63 (1:2,000, Sigma, P3737), smooth muscle actin (1:200, Epitomics, 1184-1), vimentin (1:200, Epitomics, 4211-1), K67 (1:4,000, Epitomics, 4203-1) and cleaved PARP (1:500, Epitomics, 1051-1). Primary antibodies were detected using Envision Dual Link (Dako) followed by incubation with 3,3’-diaminobenzidine tetrahydrochloride (DAB+)-chromogen (Dako). Immunohistochemistry with murine antibodies on mouse tissue was performed with UltraVision Quanto mouse on mouse horseradish peroxidase (Peroxidase). Sections were counterstained with haematoxylin and then dehydrated, cleared and mounted. Images were captured on a 3.3-megapixel QColor3 camera (QImaging), and image levels were equally scaled and auto toned in Photoshop.

**Suspension assays.** MCF10A-5E cells expressing the indicated constructs were trypsinized and plated at 400,000 cells ml⁻¹ in assay medium containing 5 ng ml⁻¹ EGF on poly-(2-hydroxyethyl methacrylate) (poly-HEMA)-coated tissue culture plates. At the indicated time points, medium was removed by centrifugation at 150g for 3 min. Cells were washed with 500 μl ice-cold PBS. For trypsinization, cells were lysed in 62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 0.01% bromphenol blue, 2.5% ethanol (0.04%) and 100 mM dithiothreitol, and whole-cell extracts were separated on 6% or 12% SDS–PAGE gel. For immunofluorescence, cells were fixed with 3.7% PFA at room temperature for 15 min, permeabilized with 0.3% Triton X-100 in PBS, and then processed for immunofluorescence as described above. For live-cell imaging, MCF10A-5E cells expressing JUND–Venus and RFP1–KRT5 were cultured on poly-HEMA-coated culture dishes with a fused glass coverslip (Matsucell). Time-lapse imaging was performed as described above.

**Flow cytometry.** Cells were dissociated with Accutase (Invitrogen) for 1 h at 37°C, fixed with 3.7% PFA, permeabilized with ice-cold methanol, and stored at −20°C. Cells were blocked with 1× western blocking reagent (Roche) in PBS-T for 30 min and incubated 1 h at room temperature with the following primary antibodies: Jun D (329; 1:100, Santa Cruz, sc-74), keratin 5 (1:2,000, Covance, SIG-3475), tenasin (BC-24; 1:2,000, Sigma, T2551), cleaved caspase-3 (Asp175; 1:200, Cell Signaling, number 9661), cleaved PARP (Asp214; 1:200, BD Pharmingen, 552597), HA (3F10; 1:200, Roche, number 11815016001). Cells were washed in PBS-T, incubated for 1 h at room temperature with Alexa Fluor 488-, phycoerythrin- and Alexa Fluor 647-conjugated secondary antibodies (Invitrogen and Jackson ImmunoResearch), and counterstained with DAPI. As collapsed cells are probably lost during this dissociation and staining procedure (Supplementary Fig. 5a), the flow cytometry quantification of KRT5 fluorescence is probably an underestimate.

Cells were analysed on a BD FACScalibur flow cytometer, equipped with 407 nm, 488 nm, 561 nm and 637 nm lasers (BD Biosciences). For DAPI, data were collected with the 407 nm laser and 455/30 or 450/50 nm band-pass filter. For Alexa Fluor 488, data were collected with the 488 nm laser and 530/30 nm band-pass filter. For phycoerythrin, data were collected with the 488 nm laser and 585/42 nm band-pass filter. For RFP, data were collected with the 561 nm laser and 580/20 nm band-pass filter. For Alexa Fluor 647, data were collected with the 637 nm laser and 666/27 or 661/16 nm band-pass filter. After acquisition, flow cytometry data were analysed with Flowjo software.

**Small-sample cDNA amplification.** Outer ECM-attached cells and inner cells (~50 each) were microdissected separately at day 6 of morphogenesis, amplified in quadruplicate by poly(A) PCR as previously described^{6,23}, and hybridized to HumanRef-8 Expression BeadChips (Illumina). The ratio of inner/outer expression was calculated with amplification standard error calculated by error propagation.

**Intraductal injections.** Luciferase- and inducible TNC-shRNA-expressing MDA-MB-468 cells were treated with or without doxycycline for three days in culture and then suspended as single cells at a concentration of 20,000 cells ml⁻¹ in MCF10A assay medium containing 5 ng ml⁻¹ EGF. Six to ten-week-old female SCID-beige mice (Charles River) were split into treatment (with doxycycline) and control (without doxycycline) groups without formal randomization. Mice were anaesthetized with isoflurane, and surgical scissors were used to cut a small crescent incision around the nipple to expose the inguinal gland. The tip of the nipple was snipped with surgical scissors, and a Hamilton syringe with a 30-gauge blunt-ended needle was used to deliver 2 μl of the cell suspension per gland (two glands per mouse). The crescent incisions were sealed with Gluture (Fisher), and mice were allowed to recover before returning to the animal facility. Treatment and control groups were fed standard rodent diet with or without doxycycline (Harlan). No statistical method was used to predetermine sample size, which was limited to the maximum number of surgeries that could be performed on one day. The investigators were not blinded to allocation during experiments and outcome assessment, and no animals were excluded from the study. All animal work was done in compliance with ethical regulations under IACUC approval number 3945.

**Bioluminescence imaging.** Bioluminescent imaging was performed at 2, 7, 14 or 21 days post-injection on an IVIS Spectrum bioluminescence and fluorescence scanner (Caliper). During imaging, mice were anaesthetized with isoflurane and subsequently injected intraperitoneally with 150 mg D-luciferin kg⁻¹ body weight. Bioluminescence imaging was initiated 5 min after injection with 2-min exposure time until steady-state luminescence was reached. Bioluminescence photon fluxes were calculated using IVIS Imaging Spectrum Software.
**METHODS**

**Statistical analysis.** Quantitative PCR data (Figs 3a–d and 6c,d) were assessed by Student’s t-test, whose suitability was verified by the F-test for homogeneity of variances after Bonferroni correction for multiple hypothesis testing. Enrichment of the TGFBR3–JUND clusters (Fig. 4a) was assessed by the hypergeometric test. Flow cytometry data (Figs 5b,j, 6k and 8e) was assessed by Welch’s t-test, with the exception of the TNC shRNA apoptotic signatures (Fig. 8g), which were assessed by two-way analysis of variance for an interaction between TNC knockdown and the frequencies of early-versus-late apoptosis. Frequency of ductal colonization (Fig. 8f) was assessed by Fisher’s exact test. Acinar areas (Supplementary Fig. 3g) were compared by rank sum test. Cell numbers (Supplementary Fig. 3h) and RNA FISH expression frequencies (Supplementary Fig. 3j,l) were compared by Welch’s t-test. Comparison of intraductal injections (Supplementary Fig. 8d) was performed by Welch’s t-test after log transformation.

**Replication of experiments.** Immunoblot images (Figs 2b,e,h, 5g, 6i,j and Supplementary Figs 3a,d, 5b,d,e, 6a,c,e,h, 7k and 8c) are representative of 2–3 independent experiments. RNA FISH images (Figs 1c,d and 2d,f and Supplementary Fig. 3l,k) are representative of 3–5 independent hybridizations. Immunofluorescence images (Figs 4c,d,g,j,k, 5a–c,i,k, 6h,l and 7a–d,g and Supplementary Figs 2a, 3b,e, 4, 5a, 6b,d,i,l, 7c,i and 8a,b) are representative of 2–3 independent *in vitro* experiments or at least three separate fields per clinical specimen (because independent experiments on each clinical specimen could not be performed). Immunohistochemistry images (Figs 4b and 8h–k and Supplementary Fig. 8e) are representative of at least three fields per specimen. Flow cytometry plots (Supplementary Figs 5c, 6g and 7l) are representative of four independent samples.

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Supplementary Figure 1 Molecular heterogeneities among ECM-attached cells during 3D organotypic culture. Basal-like cells are seeded at clonal density in reconstituted basement membrane and proliferate as spheroids for the first several days before organizing into a pre-acinus that will ultimately hollow to form a mature acinus. Various molecular heterogeneities among ECM-attached cells emerge at the pre-acinus stage during 3D culture.1-3.
Supplementary Figure 2  Normal acinar formation requires heterogeneous JUND expression at the time when stochastic profiling was performed. (a and b) Constitutive expression of JunD at the pre-acinus stage causes acini that mature with a cribiform phenotype. MCF10A-5E cells stably expressing doxycycline (DOX)-regulated murine JunD were placed in morphogenesis and induced with 1 μg/ml DOX at day 9 (Ref. 4). Acini were fixed at day 28, stained for E-cadherin (green) and HA-tagged JunD (red), and analyzed by confocal immunofluorescence. Cells were counterstained with DRAQ5 (blue) to label nuclei. For (a), scale bar is 20 μm. For (b), data are shown as the mean ± s.e.m. of n=4 independent experiments. For source data, see Supplementary Table 3.
Supplementary Figure 3 Validation of the RFP1-Smad2 and udsVenus (P\textsubscript{JUND}) reporters. (a) Densitometry of Smad2/RFP1-Smad2 abundance normalized to the pBabe control. (b) RFP1-Smad2 translocates to the nucleus in response to TGF\textbeta-family ligands. MCF10A-5E cells transduced with RFP1-Smad2 were plated on coverslips and stimulated as indicated. Nuclear RFP1-Smad2 is highlighted with arrows. (c) Schematic of the lentiviral udsVenus (P\textsubscript{JUND}) reporter. (d) MCF10A-5E cells stably expressing udsVenus (P\textsubscript{JUND}) reporter were treated with cycloheximide for the indicated times. udsVenus (P\textsubscript{JUND}) levels were analyzed by immunoblotting. Tubulin was used as a loading control. The half-life (t\textsubscript{1/2}) of udsVenus (P\textsubscript{JUND}) was estimated to be ~15 minutes by nonlinear least-squares curve fitting. (e) Correlation of udsVenus (P\textsubscript{JUND}) reporter and endogenous JUND protein. MCF10A-5E cells stably expressing udsVenus (P\textsubscript{JUND}) reporter were plated on coverslips and stained for JUND by immunofluorescence. Venus fluorescence (green) and endogenous JUND (red) were imaged together with nuclei counterstained with DAPI (blue). d.p., d.n., and s.p., denote double-positive, double-negative, and single-positive cells for udsVenus (P\textsubscript{JUND}) and JUND. (f–h) Coexpression of the RFP1-Smad2 and udsVenus (P\textsubscript{JUND}) reporters does not substantially perturb acinar morphogenesis. Acini from control cells or reporter cells were imaged by phase-contrast microscopy (f,g) or confocal microscopy (h) at day 28 of morphogenesis. Cross-sectional area (g) was calculated by digital segmentation and image analysis of phase-contrast images, and cells per acinus (h) were counted manually from optical confocal sections. Data are plotted as the median of n=4 independent samples (g) with significance assessed by rank sum test or mean ± s.e.m. of n=10 acini (h) with significance assessed by Welch’s two-sided t test. (i–l) TGF\textbeta3 and JUND expression frequency is comparable in reporter cells. Data are plotted as the mean ± s.e.m. of n=4 (j left), n=5 (j right, I right), or n=9 (I left) independent experiments with significance assessed by Welch’s two-sided t test. Control expression frequencies are reprinted from Fig. 1e. For (a) and (d), tubulin was used as a loading control. For (b), (e), (i), and (k), scale bar is 20 μm. For (f), scale bar is 200 μm. For source data, see Supplementary Table 3.
Supplementary Figure 4  TGFBR3 and JUND expression reciprocally map to KRT5 in multiple cases of premalignancy with basal-like features. (a–c) Expression of TGFBR3 and KRT5 proteins is mutually exclusive in ER-negative premalignant lesions. Similar results were obtained for the ten other TGFBR3-expressing cases in the collection of basal-like premalignant lesions. (d–f) Expression of JUND and KRT5 proteins is correlated in ER-negative DCIS. Similar results were obtained for the six other cases of DCIS in the collection of basal-like premalignant lesions. (g–i) Expression of JUND and KRT5 is anticorrelated in peripheral regions of clinging intraductal carcinoma. Similar results were obtained for the 14 other cases of clinging carcinoma in the collection of basal-like premalignant lesions. (j–l) Local inversion of the KRT5-JUND correlation in detached DCIS. Similar results were obtained for the two other cases where KRT5 was observed in both the DCIS and clinging carcinoma regions. Paraffin sections from three independent basal-like DCIS were stained for KRT5 (green) and TGFBR3 (red, a–c) or JUND (red, d–l) and imaged by widefield immunofluorescence. Nuclei were counterstained with DAPI (blue). Single-color fluorescence images are pseudocolored in the first two subpanels to highlight quantitative differences in immunoreactivity. Correlated and anticorrelated regions of expression are indicated with arrows and rectangles respectively. Note in (f) that high JUND immunoreactivity is excluded from the nucleus in KRT5-positive cells. Hematoxylin-eosin stains from the same cases are shown for comparison. Scale bar is 20 μm.

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Supplementary Figure 5  Cellular and molecular changes associated with keratinization of basal-like breast epithelia. (a) Collapse of JUND\textsuperscript{−}–KRT5\textsuperscript{K} as cellular dust. MCF10A-5E cells were placed in suspension for 48 hr. Cells were fixed and stained for KRT5 (green) and JUND (red), and analyzed by confocal immunofluorescence. Cells were counterstained with DAPI (blue) to label nuclei. Note the shrunken appearance of the JUND\textsuperscript{−}–KRT5\textsuperscript{K} cell on right. (b) Flow cytometry of MCF10A-5E cells in suspension for the indicated times. The high KRT5 cells were gated as KRT5\textsuperscript{+}–DAPI\textsuperscript{−}, and keratinized cells were gated as KRT5\textsuperscript{+}–DAPI\textsuperscript{+}. (c) KRT14, KRT15, and E-cadherin are upregulated together with KRT5 during detachment. MCF10A-5E cells were placed in suspension for the indicated times. (d and e) JUND does not affect KRT5 upregulation. MCF10A-5E cells were transduced with doxycycline (DOX)-inducible JUND-HA (d) or shJUND or shGFP control (e) and placed in suspension culture for the indicated times. For (a), scale bar is 10 μm. For (b), (d), and (e), cells were immunoblotted for the indicated proteins with Hsp90, tubulin, and actin used as loading controls, and n.d. denotes not detectable.
**Supplementary Figure 6** Loss of RPS6 phosphorylation is necessary and sufficient for keratinization of basal-like breast epithelial cells. (a–d) KRT5 upregulation and keratinization requires RPS6. MCF10A-5E cells were transduced with doxycycline (DOX)-inducible shRPS6 (two hairpins: hp #1 and hp #2) and placed in suspension culture for the indicated times. (e–g) Validation of a DOX-inducible constitutively active S6K (E389-ΔCT). Cells were treated with 1 μg/ml DOX for 24 hr and analyzed for the indicated proteins by immunoblotting or fixed and stained for p-RPS6 (red) and HA-tagged S6K (E389-ΔCT) (white) and analyzed by confocal immunofluorescence or flow cytometry. (h and i) Loss of RPS6 phosphorylation causes keratinization in attached cells. MCF10A-5E cells were treated with 10 nM PD325901 (a MEK inhibitor), 20 nM BEZ235 (a dual PI3K-mTOR inhibitor), 40 nM Temsirolimus (a TORC1 inhibitor), or 20 μM AT7867 (a dual S6K-Akt inhibitor), or DMSO (Control) for 30 hr. For (a), (c), (e), and (h), cells were analyzed for the indicated proteins by immunoblotting with Hsp90, tubulin, and actin used as loading controls. For (b), (d), and (i), cells were fixed at the indicated times and stained for KRT5 (green), shRPS6 (red RFP coexpression; b and d) or JUND (red; i) and analyzed by widefield or confocal immunofluorescence. Cells were counterstained with DAPI (blue) to label nuclei. For (b), (d), (f), and (i), scale bar is 20 μm.
Supplementary Figure 7 Inversion of the KRT5–JUND correlation tracks with local TNC expression in suspension culture and in multiple cases of clinging carcinoma. (a and b) Heterogeneous cell-intrinsic expression of TNC within breast carcinomas from the Human Protein Atlas. (c–h) Paraffin sections from six independent cases of basal-like DCIS were stained for KRT5 (green), JUND (red), and TNC (white) and imaged by widefield immunofluorescence. Note that KRT5+–TNC+–JUND– cells are in direct apposition with cells that were KRT5––TNC––JUND+. Hematoxylin-eosin stains from the same cases are shown for comparison. (i) Late stages of keratinization are associated with TNC upregulation. Cells were fixed at 24 hr, stained for KRT5 (green) and JUND (red), and TNC (white) and analyzed by confocal immunofluorescence. Cells were counterstained with DAPI (blue) to label nuclei. (j) TNC expression increases slightly during ECM detachment. Cells were analyzed for TNC by quantitative PCR. (k) TNC protein is upregulated during ECM detachment. Cells were analyzed for TNC by immunoblotting with Hsp90 used as a loading control. (l–n) Expression frequency of TNC determined by flow cytometry of total cells (l,m) or keratinized cells (KRT5+/–DAPI+, n). For (l–n), MCF10A-5E cells were placed in culture dishes or in suspension for the indicated times. For (j,m,n), data are shown as the mean ± s.e.m. of n=4 independent biological samples. For (a) and (b), scale bar is 100 μm. For (c–h), scale bar is 20 μm. For (i), scale bar is 10 μm. For source data, see Supplementary Table 3.
Supplementary Figure 8  Response of basal-like breast cancer cell lines to S6K inhibition, detachment, and injection into the mammary duct. (a and b) Loss of RPS6 phosphorylation causes keratinization in attached basal-like breast cancer cell lines. MDA-MB-468 cells (a) and MCF10DCIS.com cells (b) were treated with 10 nM PD325901, 20 nM BEZ235, 40 nM Temsirolimus, or DMSO (Control) for 30 hr. Cells were fixed and stained for KRT5 (green) and JUND (red) and analyzed by widefield immunofluorescence. Cells were counterstained with DAPI (blue) to label nuclei. (c) Changes in RPS6 phosphorylation, JUND, and KRT5 in MCF10DCIS.com cells placed in suspension for the indicated times. JUND appears as both short (JUNDs) and long (JUNDl) forms. Cells were analyzed by immunoblotting for the indicated proteins with Hsp90, tubulin, and actin used as loading controls. (d) Quantification of in vivo bioluminescence at two days post-injection of MDA-MB-468 cells. Data are plotted as the log-transformed mean ± s.e.m. of photon counts from n=17 (−DOX) or n=18 (+DOX) glands per group with significance assessed by Welch’s two-sided t test after log transformation. (e) TNC knockdown alters the spectrum of apoptosis caused by long-term detachment of MDA-MB-468 cells. Cleaved caspase-3 cells were quantified by flow cytometry after 4 days of suspension culture and separated into early apoptotic (G1–G2/M) and late apoptotic (Sub-G1) subpopulations by DAPI staining. Data are shown as the mean ± s.e.m. of n=4 independent biological samples. DOX-dependent alteration of the apoptotic subpopulations was assessed by two-way ANOVA. (f) Representative hematoxylin and eosin staining (left) and immunohistochemistry against Ki67 (proliferation marker; middle) or cleaved PARP (apoptotic marker; right) in an MDA-MB-468 intraductal xenograft. (g) DOX-treated shTNC tumors express TNC and thus have escaped knockdown. Paraffin sections from MDA-MB-468 intraductal xenografts were stained with hematoxylin and eosin (left) or TNC by immunohistochemistry (middle and right). The strong staining for TNC protein is highlighted with arrows. Scale bars are 20 µm (a, b, and g right) and 80 µm (f and g left and middle). For source data, see Supplementary Table 3.
**Supplementary Figure 9** Uncropped scans with size markers for immunoblot data.
Supplementary Figure 9 continued Uncropped scans with size markers for immunoblot data.
Supplementary Figure 9 continued Uncropped scans with size markers for immunoblot data.
Supplementary Video Legends

Supplementary Video 1 Dynamically coupled JUND–TGFβ signaling. An MCF10A-5E acinus stably expressing udsVenus (P\textsubscript{JUND}) and RFP1-Smad2 was illuminated at a fixed optical plane every 15 minutes for 18 hours. udsVenus (P\textsubscript{JUND}) (Top, left: gray; right: pseudo-color; Bottom, right: green) represents the endogenous activity of JUND promoter and RFP1-Smad2 (Bottom, left: gray; right, red) represents the activity of TGFβ signaling. The ECM-attached cell showing dynamic changes is indicated with an arrow.

Supplementary Table Legends

Supplementary Table 1 Summary of clinical cases of normal breast epithelium and basal-like premalignancies.

Supplementary Table 2 Proteome-wide survey of ECM heterogeneity and localization in breast carcinomas.

Supplementary Table 3 Statistics source data.

Supplementary Data File 1 Source code for the MATLAB simulations of the TGFBR3–JUND circuit in Fig. 3f-h.

Supplementary Data File 2 Source code for the NetLogo simulations of clinging carcinoma in Fig. 7e,.

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Supplementary Note S1 Extended details and validation of the computational models

Ordinary differential equations model of the TGFBR3–JUND circuit

We modeled the dynamics of TGFBR3 mRNA, JUND mRNA, TGFBR3 protein, JUND protein, active TGFBR3* (as read out by the RFP1-Smad2 reporter), and the udsVenus reporter through the following system of six ordinary differential equations:

\[
\begin{align*}
\frac{dTGFBR3}{dt} &= k_{basal} - f1 \frac{[TGFBR3]^{\text{alt}} + (IC50_{TGFBR3})^{\text{alt}}}{[JUND]^{\text{alt}} + (IC50_{JUND})^{\text{alt}}} - k_{degRNA}[TGFBR3] \\
\frac{d[JUND]}{dt} &= k_{basal} - f2 \frac{[TGFBR3]^{\text{alt}} + (IC50_{TGFBR3})^{\text{alt}}}{[JUND]^{\text{alt}} + (IC50_{JUND})^{\text{alt}}} - f3 \frac{[JUND]}{[JUND]^{\text{alt}}} - k_{degRNA}[JUND] \\
\frac{dTGFBR3}{dt} &= k_{translation}[TGFBR3] - k_{degTGFBR3}[TGFBR3] - k_{activation}[TGFBR3] \\
\frac{d[JUND]}{dt} &= k_{translation}[JUND] - k_{degJUND}[JUND] \\
\frac{dTGFBR3^*}{dt} &= k_{activation}[TGFBR3] - k_{degTGFBR3^*}[TGFBR3^*] \\
\frac{d[udsVenus]}{dt} &= k_{basal}[JUND] - k_{degudsVenus}[udsVenus]
\end{align*}
\]

The negative feedbacks relating JUND to TGFBR3 (Fig. 3a), TGFBR3* to TGFBR3 (Fig. 3d), TGFBR3* to JUND (Fig. 3c), and JUND to JUND (Fig. 3c) were modeled as Hill functions. For simplicity, we did not assume any cooperativity in the negative feedbacks (nH = 1), and IC50 values were set to nonsaturating values (IC50_{TGFBR3} = 100, IC50_{JUND} = 100). The relative feedback strengths (f1, f2, and f3) were adjusted manually to capture the experimentally observed dynamics (Fig. 3f,g; f1 = 7, f2 = 9, f3 = 5).

The degradation rates of TGFBR3, JUND, and udsVenus were estimated by treating cells with cycloheximide and quantifying protein loss by immunoblotting (Fig. N1 and Supplementary Fig. 3d). These experiments yielded half-life estimates of 14 min for TGFBR3, 1.86 hr for JUND, and 15 min for udsVenus.

Using the following relationship between degradation rate and half-life:

\[
k_{\text{degradation}} = \frac{\ln(2)}{t_{1/2}}
\]

We arrived at the following degradation rate estimates: k_{degTGFBR3} = 3.0 hr^{-1}, k_{degJUND} = 0.37 hr^{-1}, and k_{degudsVenus} = 2.8 hr^{-1}. Degradation of TGFBR3* was assumed to be equal to that of TGFBR3. As the half-lives of TGFBR3 and JUND mRNA are comparable (t_{1/2} = 2–4 hr)^{1,2}, we assumed that k_{degRNA} = 0.23 hr^{-1} (t_{1/2} = 3 hr). We obtained basal transcription (k_{basal} = 4 hr^{-1}) and translation rates (k_{translation} = 100 mRNA^{-1} hr^{-1}) as representative values from proliferating mammalian cells^{3}. The basal activation rate of TGFBR3 was calculated using k_{degTGFBR3} and the steady-state ratio of nuclear-cytoplasmic
fluorescence of the RFP1-Smad2 reporter in unstimulated cells (assuming that the reporter is directly proportional to the relative activation of TGFBR3; Supplementary Fig. 3b):

\[
k_{\text{activation}} = k_{\text{degTGFBR3}} \frac{[\text{TGFBR3}^*]}{[\text{TGFBR3}]_{\text{SS}}} = 3.0 \frac{1}{3} \text{ hr}^{-1} = 1.0 \text{ hr}^{-1}
\]

(8)

The perturbations in Fig. 3h were initiated by increasing \(k_{\text{activation}}\) of TGFBR3, \(k_{\text{basaltxn}}\) of TGFBR3, or \(k_{\text{basaltxn}}\) of JUND to 50% higher than the standard value for 1 hr. The code for generating Fig. 3h is available in Supplementary Data File 1.

When the model was perturbed, we found that the system response fell into five categories (Fig. N2): a) Undamped oscillations that remain in a limit cycle; b) Damped oscillations as in Fig. 3h (left); c) No oscillations, characterized by a transient activation or repression event as in Fig. 3h (right); d) Mixed oscillations, where one of the reporters oscillates but the other does not; e) Model error, where the steady-state activity of one reporter is near zero and the system no longer responds to the perturbation or returns infeasible values. Focusing on the damped oscillations that were noted upon TGFBR3 activation in the model (Fig. 3h), we performed a sensitivity analysis. For the six parameters that were not drawn from the literature or directly measured (\(f_1, f_2, f_3, nH, IC50_{\text{TGFBR3}}, IC50_{\text{JUND}}\)), we systematically perturbed the default parameter by tenfold in either direction and then assessed system behavior. This sensitivity analysis would indicate how fragile or robust the oscillatory network was to feedback parameters.

For nearly all feedback parameters, we found that the system exhibited damped or undamped oscillations for a wide range of parameters (Fig. N3). The one exception was for the relative strengths of the negative feedbacks from active TGFBR3 signaling and JUND expression to the expression of TGFBR3 mRNA, where damped oscillations were observed over a somewhat narrow window (Fig. N3e). If the three feedback terms were reasonably balanced, then oscillations were robust to any single change in feedback (Fig. N3e). However, if the collective negative regulation on JUND was changed by concurrently increasing \(f_2\) and \(f_3\), then oscillations stopped. This emphasizes the need for tight coupling between the TGFBR3 and JUND pathways.
JUND branches of the circuit (Fig. 3e) and may explain why not all matrix-attached basal breast epithelia oscillate during 3D culture.

Agent-based model of JUND, KRT5, and TNC in clinging carcinoma

The purpose of the agent-based model was to build a simplified representation of the mosaic KRT5–JUND expression patterns observed in ECM-poor regions of basal-like premalignancies. Agent-based models allow the arbitrary arrangement of “agents” (here, clinging carcinoma [CC] cells) to react subject to a user-defined rule set that is simulated in discrete time intervals6. The process of keratinization happens within hours (Fig. 5d) and is triggered before 24 hr of detachment (Fig. 5c and 8a). Therefore, we assumed that the effects of proliferation, death, and migration would be negligible over this time period. Notably, detachment-induced cell death (anoikis) of basal breast epithelia is not maximal until 48 hr or later (Supplementary Fig. 5b and Ref. 7) and epithelial proliferation is generally minimal without integrin engagement8.

The key facets of the model rule set are:

- JUND and KRT5 compete to determine survival vs. keratinization (Fig. 5e-k)
- Keratinization is irreversible after the nucleus has been lost (Fig. 5c)
- Keratinization is strongly associated with the expression of TNC (Supplementary Fig. 7n)
- Cells adjacent to TNC-expressing cells often have not keratinized (Fig. 7a,b)

Figure N3 Pairwise sensitivity analysis of the TGFBR3–JUND model in response to the transient activation of TGFBR3. Model parameters were changed from 0.1x to 10x of the values listed in Supplementary Note 1. See Fig. N2 for examples of each category of model response.
For the model, cells were seeded according to the characteristic geometry of a region of CC—multiple layers of neoplastic cells on the periphery of a hollow lumen. In addition to the geometry of Fig. 7e,f, we also tested alternative geometries with differing thicknesses of cells (Fig. N4). We consistently found strings of keratinized cells on the luminal face of the clinging region, as well as local homogeneities of JUND expression. These additional simulations indicate that our conclusions are not limited to a specific geometry of CC. Source code for the NetLogo script and tissue geometries can be found in the Supplementary Data File 2.

**Figure N4** Agent-based model predictions do not depend on the specific geometry of clinging carcinoma. (a) Elliptical and (b) open-diagonal geometries were seeded with various thicknesses of carcinoma cells and simulated as described in the manuscript. Keratinized cells (yellow) and JUND levels (red) are shown with and without the simulated role of TNC. Stretches of luminally positioned, keratinized cells are highlighted in solid boxes. Local homogeneities of JUND expression are highlighted in dashed boxes.
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