Twist1-induced dissemination preserves epithelial identity and requires E-cadherin

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Dissemination of epithelial cells is a critical step in metastatic spread. Molecular models of dissemination focus on loss of E-cadherin or repression of cell adhesion through an epithelial to mesenchymal transition (EMT). We sought to define the minimum molecular events necessary to induce dissemination of cells out of primary murine mammary epithelium. Deletion of E-cadherin disrupted epithelial architecture and morphogenesis but only rarely resulted in dissemination. In contrast, expression of the EMT transcription factor Twist1 induced rapid dissemination of cytokeratin-positive epithelial cells.

Introduction

Metastasis is the primary cause of death in breast cancer, and patient outcomes correlate negatively with the extent of metastatic spread at diagnosis (Bogenrieder and Herlyn, 2003; Polyak, 2010). Metastasis initiates with dissemination, the escape of epithelial cancer cells from the primary tumor into the surrounding stroma (Nguyen et al., 2009). As dissemination requires loss of epithelial cell–cell junctions, a change in the expression of intercellular adhesion genes could be the initiating event (Nelson, 2009; Polyak and Weinberg, 2009). Two related molecular models for dissemination have been proposed on this basis: genomic loss of cell adhesion genes (Hirohashi, 1998; Bogenrieder and Herlyn, 2003; Jeanes et al., 2008) and repression of cell adhesion genes through an epithelial to mesenchymal transition (EMT; Peinado et al., 2007; Yang and Weinberg, 2008).

These molecular models converge on the cell adhesion gene Epithelial cadherin (E-cad; Cdh1). E-cad is essential for early embryonic development (Larue et al., 1994); E-cad expression is frequently lost in human breast cancer (Berx et al., 1996); and E-cad loss in experimental cancer models accelerates metastatic progression (Derksen et al., 2006; Onder et al., 2008). However, a gap exists in our understanding of the relationship between the normal role of E-cad in adult tissues and its function during cancer metastasis. Analyses of E-cad’s requirement in adult epithelial tissues using Cre-mediated deletion (Boussadia et al., 2002) have revealed highly varied E-cad null phenotypes. Conditional E-cad deletion in the mammary gland results in cell death during lactation (Boussadia et al., 2002), whereas conditional E-cad deletion in the skin results in hyperproliferation of some cell types and premature degeneration of others (Tinkle et al., 2004). E-cad deletion in these developmental contexts is not associated with systemic dissemination.

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Nevertheless, transcriptional repression of E-cad by EMT transcription factors such as Twist1 remains a central concept in cancer metastasis (Peinado et al., 2007; Yang and Weinberg, 2008). Twist1 regulates metastasis in a mouse mammary tumor model (Yang et al., 2004), and its expression is up-regulated in both invasive lobular and invasive ductal breast cancer (Yang et al., 2004; Mironchik et al., 2005).

Collectively, previous publications have demonstrated that E-cad functions as an invasion suppressor and that induction of EMT transcription factors can accelerate malignant progression (Hirohashi, 1998; Berx and Van Roy, 2001; Bogenrieder and Herlyn, 2003; Derksen et al., 2006; Yang and Weinberg, 2008; Polyak and Weinberg, 2009). However, human breast tumors typically contain thousands of mutations in both signaling and structural genes (Wood et al., 2007; Stephens et al., 2009). These coexisting mutations obscure the contribution of individual genetic events to discrete steps in the metastatic cascade. Specifically, the presence of additional mutations in cancer cell lines has made it difficult to distinguish the individual sufficiency of E-cad deletion or Twist1 expression for dissemination. Importantly, dissemination can be induced in developmental contexts such as neural crest migration, suggesting that its molecular regulation may be distinct from other aspects of neoplasia (Barrallo-Gimeno and Nieto, 2005).

We sought to define the minimum molecular perturbations necessary to induce dissemination of normal mammary epithelial cells. To accomplish these goals, we used a combination of organotypic culture, Cre-lox–based genetic models, inducible expression systems, lentiviral gene knockdown (KD), and time-lapse imaging to test the sufficiency of E-cad deletion or Twist1 expression to induce dissemination in primary mammary epithelium. Our results demonstrate that E-cad is required for simple epithelial architecture and branching morphogenesis, but its loss is not associated with significant dissemination in 3D culture or in vivo. In contrast, Twist1 expression induces rapid epithelial dissemination. Moreover, Twist1-induced dissemination occurs without loss of epithelial gene expression and requires E-cad.

## Results

### E-cad is required for simple mammary epithelial architecture

We first assayed the acute consequences of E-cad deletion in primary mammary epithelial cells using the “organoid” assay, in which mammary ducts are explanted into 3D ECM (Matrigel; Ewald et al., 2008; Fig. 1 A). In basal medium without growth factors, normal organoids form polarized cysts (Ewald et al., 2008; Fig. 1, A and B). We isolated organoids from mice carrying floxed E-cad alleles (Boussadia et al., 2002) and a ubiquitously expressed, tamoxifen-inducible Cre recombinase (Badea et al., 2003; Cre-ER;E-cadfl/fl mice). Organoids from the same mouse were divided into a control group and an experimental group in which E-cad deletion was induced with 50 nM tamoxifen.

Control organoids efficiently formed polarized cysts, with E-cad localized to lateral points of cell–cell contact and zona occludens 1 (ZO-1) localized in apical puncta (58.2%; Fig. 1, B, D, E, and E’). In contrast, E-cad– organoids either failed to establish simple epithelial architecture (66%) or transiently established and then lost simple epithelial architecture (33%; Fig. 1, C and D). E-cad– organoids had a multilayered organization and lacked morphologically evident lumens (Fig. 1, E and F). Interestingly, a subset of lateral cell–cell contact surfaces within E-cad– organoids had continuous ZO-1 staining (Fig. 1, F and F’).

Immunofluorescence (Fig. 1 F) and Western blotting (Fig. 1 G) confirmed loss of E-cad protein by day 6 in culture. E-cad loss coincided with a reduction in αE-catenin and β-catenin (Fig. 1 G) but not with a significant change in neural cadherin (N-cad; Fig. S1, A and B). By immunofluorescence, αE-catenin was absent from most internal cell–cell contact surfaces (Fig. S1, C–C’).

### E-cad– cells are excluded from regions of simple epithelial organization but rarely disseminate

We next used time-lapse microscopy to observe the cell dynamics driving loss of simple organization after E-cad deletion. We monitored recombined cells using the genetically encoded Cre biosensor mT/mG (Muzumdar et al., 2007). Without Cre activity, all cells in mT/mG mice express a membrane-localized red fluorescent protein. Cre activity excises the red gene and induces heritable expression of a membrane-localized green fluorescent protein. We could thereby distinguish in real-time the behaviors of E-cad– (red) versus E-cad– (green) cells. Loss of simple epithelial architecture in E-cad– organoids correlated with a reduction in luminal volume (Fig. 1 H and Video 1), consistent with a loss of tight junctions. This tissue-level change was accompanied by a change in E-cad– cell shape from simple columnar to round (Fig. 1, H’ and H”). Round cells shifted internally, inducing a transition from a single to multiple luminal epithelial cell layers (Fig. 1, H’ and H”, arrowheads).

We next monitored whether E-cad deletion was sufficient to induce dissemination into the ECM. Normal epithelial cysts maintain a smooth basal surface with the ECM (Fig. 2 A). Conversely, E-cad– cells accumulated on the basal surface and collectively migrated into Matrigel as single file columns (Fig. 2, B, B’, G, and G’) and as disorganized masses (Fig. 2, E, E’, F, and F’). Single file migration initiated from basally positioned E-cad– cells that rounded up but maintained cell–cell contact (Fig. 2 C). As additional cells changed shape, the initiating cell migrated further into the ECM and led a column of closely connected E-cad– cells. However, despite contact with the ECM and high motility, E-cad– cells rarely disseminated into the matrix. Each organoid consists of 300–500 cells; yet, on average, only one cell disseminated from each E-cad– organoid (n = 206 organoids imaged by time-lapse microscopy across nine biologically independent replicates). Most E-cad– cells remained adherent to other epithelial cells. The few E-cad– cells that did disseminate were rounded, migrated minimally, and had no detectable membrane protrusions.
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apical to polarized E-cad cells (Fig. 2, D–F). The basally positioned E-cad cells were frequently observed past the cell-derived basement membrane (laminin 332 immunofluorescence; Fig. 2, G and G'). E-cad deletion blocked cyst formation. E-cad deletion induced loss of simple epithelial architecture. (A) E-cad deletion was induced in half of Cre-ER;E-cad<sup>fl/fl</sup> organoids with tamoxifen. (B) Control, E-cad<sup>+</sup> organoids (−Tam) formed cysts. (C) E-cad<sup>−</sup> organoids (+Tam) failed to form cysts (28/42 movies across three biological replicates) or transiently established and then lost lumens (14/42 movies). (D) E-cad deletion blocked cyst formation. n, total number of organoids; r, number of biological replicates. Error bars indicate SD. ***, P = 0.0004, two-tailed Student's t test with equal variance. (E) Control organoids formed cysts with enrichment of E-cad and ZO-1 along apicolateral membranes (E'). (F) E-cad<sup>−</sup> organoids were multilayered, lacked E-cad immunoreactivity, and displayed abnormal ZO-1 localization. Arrow indicates rare E-cad<sup>+</sup> cells. (G) By Western blot, E-cad deletion (+Tam) resulted in complete loss of E-cad protein and significant reductions in αE-catenin and β-catenin (see also Fig. S1, A and B). Whole cell lysate samples were loaded for equal protein based on BCA analysis. (H) The Cre biosensor mT/mG was used to observe E-cad<sup>−</sup> cell behaviors by confocal microscopy [Video 1]. Cre', E-cad<sup>+</sup> cells (green) changed shape, from columnar to round, before shifting apically (H' and H'', arrowheads). Gamma adjustments were performed in E and F to improve image clarity. Bars: (B and C) 20 µm; (E, F, and H) 10 µm.

**E-cad<sup>−</sup>** cells remain adherent despite reductions in multiple classes of intercellular junctions

To analyze the effects of mosaic E-cad loss, we used adenovirally delivered Cre (Adeno-Cre) to induce E-cad deletion in a subset of cells within E-cad<sup>fl/fl</sup>,mT/mG organoids (Fig. 2 D). We confirmed that green, Cre<sup>+</sup> cells stained negative for E-cad protein (Fig. 2, E and E') and that loss of E-cad was accompanied by loss of β-catenin from the membrane (Fig. 2, F and F'). In these genetic mosaic organoids, E-cad<sup>−</sup> cells were observed both basally at the cell–ECM interface and in interior positions apical to polarized E-cad<sup>+</sup> cells (Fig. 2, D–F). The basally positioned E-cad<sup>−</sup> cells were frequently observed past the cell-derived basement membrane (laminin 332 immunofluorescence; Fig. 2, G and G'). E-cad<sup>−</sup> cells within the same organoid localized β-catenin to points of cell–cell contact and were inside the basement membrane (Fig. 2, E–G).

We next sought to identify adhesion systems that could allow epithelial cells to remain adherent despite loss of E-cad and membrane-localized β-catenin. Desmosomes represent a major class of intercellular junctions in mammary epithelial cells (Bissell and Bilder, 2003). However, E-cad inhibition can
induce reductions in both desmosomes and tight junctions (Gumbiner et al., 1988). We therefore used transmission electron microscopy (TEM) to quantify the effect of E-cad loss on intercellular junctions in organoids from E-cad+ and E-cad− littermates. We induced recombination with Adeno-Cre and focused our analysis on basally positioned cells (Fig. 2 H). E-cad− cells typically lacked both punctate ZO-1 immunoreactivity (Fig. 1 F) and ultrastructurally identifiable tight junctions. Compared with normal E-cad+ epithelium, E-cad− epithelium also had a statistically significant, almost eightfold reduction in desmosomes (Fig. 2 I). However, we still detected small desmosomes connecting E-cad− cells, even within single file migration columns (Fig. 2 J and J'). Collectively, our data reveal that loss of E-cad results in loss of simple epithelial architecture, reductions
in multiple classes of cell–cell junctions, and both apical and basal exclusion of E-cad− cells from E-cad+ simple epithelium. However, E-cad loss was not sufficient for robust single cell dissemination into the 3D ECM.

**E-cad is required for branching morphogenesis in 3D culture**

Normal mammary branching morphogenesis begins with a transition from simple to multilayered architecture and a concurrent reduction in apicobasal polarity and intercellular junctions (Ewald et al., 2008, 2012). Therefore, it was plausible that E-cad− cells could participate in branching morphogenesis. We thus assayed the effects of E-cad deletion under culture conditions that use FGF2 to induce branching morphogenesis (Ewald et al., 2008; Fig. 3 A).

We first induced E-cad deletion in most epithelial cells using Adeno-Cre and monitored effects on branching by time-lapse microscopy. Control E-cad+ organoids underwent normal branching morphogenesis (Video 2). Specifically, they initiated and elongated numerous mammary buds and main-stem ducts, and maintained a smooth border with the ECM. In contrast, E-cad− organoids did not undergo branching morphogenesis and instead rapidly developed a disorganized and uneven basal epithelial surface (Fig. 3 C; Video 2). Cells at the basal ECM border were rounded and displayed extensive, uncoordinated motility (Fig. 3 C'). These cells were Cre biosensor+ (green) and E-cad−

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**Figure 3. Loss of E-cad inhibited branching morphogenesis and induced epithelial disorganization in 3D culture.** (A) E-cad+/+;mT/mG and E-cad+/E-cad−;mT/mG organoids were isolated, recombination was induced with Adeno-Cre, and branching morphogenesis was induced with FGF2. (B) E-cad+ organoids completed branching morphogenesis [Video 2]. (C) E-cad− organoids developed a disorganized basal surface composed of rounded cells (C'; Video 2). (D) This disorganized surface morphology was observed in 94% of E-cad− organoids. n, number of time-lapse movies; τ, number of biological replicates. (E and F) Basally positioned cells were green [Cre+], E-cad− [E], and β-catenin− [F]. (G and H) In genetic mosaic organoids with a mixture of E-cad+ and E-cad− cells, E-cad+ cells (red) were observed to initiate new buds [arrowheads; H'; Video 3]. Bars: [B and C] 20 µm; [E, F, and H] 10 µm.
by immunofluorescence (Fig. 3, E and E’) and lacked β-catenin at points of cell–cell contact (Fig. 3, F and F’).

We next induced genetic mosaic E-cad deletion and observed some normal, smooth epithelial buds emerging from disorganized cell surfaces. We hypothesized that these buds were composed of E-cad+ cells that had escaped recombination (Fig. 3 G). Consistent with this model, we observed groups of red, E-cad+ cells coalesce and initiate new buds from within large disorganized groups of green, E-cad− cells (Fig. 3, H and H’; and Video 3). Our data demonstrate that E-cad− cells remain motile and adherent but fail to incorporate into epithelial buds. Furthermore, in genetic mosaic mixtures, E-cad+ cells can initiate buds from predominantly E-cad− organoids (Fig. 3, G and H).

E-cad− cells are excluded from polarized ducts and the body cell compartment of the terminal end bud (TEB) in vivo

Our genetic mosaic analysis in 3D culture revealed that E-cad− cells lost simple epithelial architecture and most intercellular junctions but remained adherent to each other. However, the presence of E-cad− cells in an organoid did not prevent the initiation of E-cad+ epithelial buds. Accordingly, we predicted that, in vivo, genetic mosaic E-cad deletion would result in exclusion of E-cad− cells from regions of active branching morphogenesis and accumulation of disorganized E-cad− cell groups both apically and basally. To test this prediction, we isolated organoids from control E-cadfl/fl; mT/mG mice and from E-cad0/0; mT/mG mice, induced mosaic recombination with Adeno-Cre, and transplanted the organoids into contralateral, cleared mammary fat pads of 3-wk-old NOD/SCID mice (Fig. 4, A and D, respectively). Glands were harvested and analyzed 6 wk after transplantation.

Mammary ducts during puberty are elongated by specialized epithelial structures known as TEBs (Williams and Daniel, 1983). TEBs are composed of a single, basally positioned layer of cap cells and multiple, apically positioned body cell layers (Hinck and Silberstein, 2005). Cap cells give rise to myoepithelial lineages, whereas body cells give rise to luminal epithelial lineages. Only body cells express E-cad (Daniel et al., 1995). Ductal outgrowths from control, genetic mosaic mT/mG transplants had both red and green cells in the body and cap cell regions of the TEB (Fig. 4, B and B’). In the luminal and myoepithelial cell layers of polarized ducts (Fig. 4, C and C’). In contrast, outgrowths from genetic mosaic E-cad0/0; mT/mG transplants displayed a striking exclusion of E-cad− cells from the body cell region of the TEB (Fig. 4, E and E’) and from the luminal layer of ducts (Fig. 4, F and F’). Myoepithelial cells were red and green in E-cad0/0; mT/mG genetic mosaic outgrowths (Fig. 4 E’, green arrowheads), but myoepithelial cells express P-cadherin instead of E-cad (Daniel et al., 1995). Despite their exclusion from polarized ducts and body cells, E-cad− cells were observed in vivo at 6 wk after transplantation. Groups of E-cad− cells were detected on the basal surfaces of polarized E-cad+ ducts in the gland periphery (Fig. 4, F and F’) and on the basal surfaces of polarized E-cad− epithelium near the transplantation site (Fig. 4, G and G’). We also observed small clusters of exclusively E-cad− cells, surrounded by myoepithelial cells (smooth muscle actin− [SMA]; Fig. 4, H and H’). Consistent with our 3D culture data, green, Cre+ cells in vivo were validated by antibody staining to lack membrane-localized E-cad (Fig. S1, D–F) and β-catenin (Fig. S1G).

We next tested the in vivo consequences of E-cad deletion in polarized mammary epithelium. We isolated and transplanted organoids from Cre-ER; E-cad0/0; mT/mG mice into cleared mammary fat pads, waited 6 wk for mature ductal outgrowths, and induced E-cad deletion by tamoxifen injection (Fig. 4 I). Glands were harvested and analyzed 2–6 wk after tamoxifen injection. E-cad− cells (by immunofluorescence) were observed apically inside duct lumens (Fig. 4, J and J’) and basally as single cells or disorganized groups on duct surfaces (Fig. 4, J and J’).Basally positioned E-cad− cells were typically still surrounded by myoepithelial cells. We conclude that E-cad is required in mammary epithelial cells to establish and maintain simple epithelial architecture and to initiate and elongate new buds, both in 3D culture and in vivo. E-cad− cells are viable in both contexts but extrude apically and basally from polarized epithelium and fail to contribute to mammary branching morphogenesis in the luminal cell compartment.

Twist1 induces robust cell dissemination of otherwise normal primary epithelial cells

Our data reveal that E-cad loss is not sufficient for robust cell dissemination in 3D culture or in vivo. This conclusion has implications for our conceptual understanding of EMT, as repression of E-cad is considered a core effector of EMT (Yang et al., 2004; Peinado et al., 2007; Vesuna et al., 2008; Thiery et al., 2009). The bHLH transcription factor Twist1 has emerged as a candidate regulator of EMT in cancer and is thought to act through regulation of E-cad (Yang et al., 2004; Vesuna et al., 2008; Yang and Weinberg, 2008). We sought to test the hypothesis that acute expression of Twist1 would induce epithelial dissemination.

We isolated organoids from mice carrying a ubiquitously expressed reverse tetracycline transactivator (rtTA) and a Tet-responsive Twist1 allele (Fig. 5 A, CMV::rtTA; TRE-Twist1; Tran et al., 2012). In basal medium, control organoids maintained a smooth basal surface (Fig. 5 B). In contrast, Twist1 expression induced rapid dissemination of prosurform, individual cells out of the epithelium as early as 24 h after Twist1 induction (Fig. 5, C and C’; and Video 4). We next tested the relationship between branching morphogenesis and dissemination. In FGF2-containing medium, control organoids branched efficiently (Fig. 5 D). In contrast, Twist1 expression inhibited FGF2-induced branching morphogenesis in 99% of organoids and induced robust dissemination in 97% of organoids (Fig. 5, D–F; and Video 5). In the presence of FGF2, disseminated cells proliferated to form secondary epithelial sites within the ECM (Fig. 5 E’). Immunofluorescent staining for cell type–specific cytokeratins revealed that both luminal epithelial (K8+) and myoepithelial (K14+) cells disseminated in response to Twist1 induction (Fig. 5 G). Interestingly, both cells within the epithelial structure and disseminated cells displayed nuclear Twist1.
immunoreactivity (Fig. 5, H and H'). The Twist1+ epithelial group displayed abnormal internal localization of myoepithelial cells (SMA+) and basement membrane (laminin 332) and corresponding gaps in basal myoepithelial and basement membrane coverage (Fig. 5 I).

Epithelial cell behaviors are restored when Twist1 expression is turned off
We next tested the consequences of transient expression of Twist1. We induced Twist1 for 48 h and then turned off Twist1 by removing doxycycline (Fig. 6 A). In basal medium, disseminated single cells stopped migrating within 48 h of doxycycline removal (Fig. 6, B and B'; and Video 6). In FGF2-containing medium, organoids initiated branching morphogenesis within 70 h of doxycycline removal (Fig. 6, C and D; and Video 7), and disseminated single cells were observed reintegrating with the epithelial group (Fig. 6 E). Remarkably, by day 7, the resulting branched structures had normalized epithelial organization, with internal luminal cells and basally positioned myoepithelial cells (Fig. 6 F).

We conclude that epithelial cells can rapidly reestablish normal developmental programs, such as branching morphogenesis, when Twist1 expression ceases.
Twist1-induced dissemination is cell autonomous

We demonstrated that ubiquitous Twist1 activation induced rapid epithelial dissemination. We next sought to test whether a single Twist1+ cell could escape a mostly Twist1− epithelium. We reasoned that Twist1+ cells could serve as a barrier to dissemination. Alternatively, Twist1+ cells could induce the dissemination of neighboring Twist1− cells. To achieve mosaic activation of Twist1, we used a Lox-Stop-Lox-rtTA (R26::LSL-rtTA [Belteki et al., 2005]) and varying titers of Adeno-Cre to modulate the fraction of cells capable of activating Twist1. We again used the mT/mG reporter (Muzumdar et al., 2007) to distinguish between Cre+rtTA+ (green) and Cre−rtTA− (red) cells. We monitored the resulting genetic mosaic tissue for dissemination in our branching morphogenesis assay (Fig. S2 A).

Figure 5. Twist1 induced robust dissemination of normal epithelial cells. (A) Organoids were isolated from CMV::rtTA;TRE-Twist1 mice, and Twist1 was induced in half of the organoids with doxycycline. (B and C) In basal medium, control organoids maintained epithelial organization (B), whereas Twist1 expression induced robust dissemination (C; Video 4). Disseminating cells (red arrowheads) migrated away from the epithelium with extensive protrusions (C'). (D and E) In FGF2-containing medium, control organoids completed branching morphogenesis (D), whereas Twist1 expression blocked branching and induced robust dissemination (E; Video 5). Red arrowheads in E indicate disseminated cells. With FGF2, disseminated cells proliferated to form secondary epithelial sites (blue arrows; E′; 6/9 biological replicates). (F) Less than 1% of Twist1− organoids branched (***, P = 0.0006, two-tailed Student’s t test with equal variance), whereas 97% disseminated (****, P = 4 × 10−7, two-tailed Student’s t test with equal variance). n, total number of organoids; r, number of biological replicates. Error bars indicate SD. (G) Both luminal (K8+; red arrows) and myoepithelial (K14+; green arrows) cells disseminated. (H) Both disseminated single cells and cells within the main epithelial group were Twist1+. (I) Myoepithelial cells (SMA+) and basement membrane (laminin 332; arrows) were inappropriately localized to the organoid interior. Gamma adjustments were performed in H, H′, and I to improve image clarity. Bars: (B–E) 20 µm; (G–I) 10 µm.
Twist1 is sufficient to induce local dissemination in vivo

Our 3D culture data predicted that Twist1 would induce epithelial dissemination in vivo. To test this prediction, we transplanted genetic mosaic organoids containing a mixture of red, Twist1− and green, Twist1+ cells into cleared mammary fat pads of 3-wk-old NOD/SCID mice (Fig. S2 F). Twist1 was induced in culture and maintained in vivo using doxycycline feed. Consistent with our 3D culture data, we observed local dissemination of green, Twist1+ cells into the surrounding stroma (Fig. S2, G−G′). We did not detect dissemination of red, Twist1− cells. Importantly, we observed groups of 10−20 green, Twist1+ epithelial cells in the stroma (Fig. S2, H and H′). We hypothesize that these groups represent secondary epithelial sites formed by Twist1-induced dissemination.
from disseminated Twist1+ cells. We conclude that Twist1 expression is sufficient for epithelial dissemination in 3D culture and in vivo.

**Twist1 induces dissemination without complete loss of adherens junction components**

Our genetic analyses revealed that loss of E-cad and expression of Twist1 induced distinct cell behaviors and tissue-level phenotypes. E-cad deletion resulted in loss of polarized epithelial architecture, whereas Twist1 expression induced dissemination of luminal and myoepithelial cells (Fig. 7, A–G). We next used immunoblotting to compare levels of cell–cell adhesion proteins between normal (−Dox) and Twist1-expressing (+Dox) tissue. We observed significant reductions in E-cad, αE-catenin, β-catenin, and N-cad (Fig. 7 H and Fig. S3, A and B) but detected protein in all cases. This reduction in N-cad is inconsistent with a cadherin switch model in which increases in N-cad levels induce migration (Nieman et al., 1999). We next used immunofluorescence to localize E-cad and β-catenin during dissemination. Consistent with our Western blot results, E-cad staining appeared weaker in cells expressing Twist1. However, we observed E-cad and β-catenin localized at points of cell–cell contact in cells before dissemination (Fig. 7, I and L); E-cad and β-catenin localized to the rear of recently disseminated cells (Fig. 7, J and M); and E-cad and β-catenin localized to the membranes of cells migrating in the ECM (Fig. 7, K and N). We also observed E-cad− cells in the matrix, consistent with the dissemination of K14+ myoepithelial cells, which would not normally express E-cad. We conclude that Twist1 can induce dissemination of cells with membrane-localized adherens junction proteins.

**Twist1 induces transcriptional changes in cell–matrix adhesion but does not fundamentally alter epithelial identity**

We next sought to identify the early transcriptional changes downstream of Twist1. We isolated organoids from three CMV::rtTA;TRE-Twist1 mice and three rtTA− littermate controls.
We conclude that our data identify a novel set of genes regulated associated with at least three cancer-related gene sets (Fig. 9 B). Out of 183 DE genes, 33 were associated with curated gene sets, 13 of which we characterized as cancer related, collagen formation, ECM–receptor interactions, and ECM significant canonical pathways, all related to the cell interface gene lists from the literature (curated pathways). We found eight way databases (canonical pathways) were augmented to include regulation by calculating the absolute value of the differential expression. Because we observed discordant expression wide significance, we also performed a more general test of difference (Fig. S4 F), and nucleus.

DE genes were also associated with the cytoplasm (Fig. S4 E), plasma membrane lar space, and proteinaceous ECM (Fig. 8 F). DE genes were also significantly more DE genes than expected in GO cell migration, cell projections, and ECM organization (Fig. 8 D). Associated with cell–substrate, not cell–cell, adhesion and with cell migration, cell projections, and ECM organization (Fig. 8 D). We observed significantly more DE genes than expected in GO cellular component categories for extracellular region, extracellular space, and proteinaceous ECM (Fig. 8 F). DE genes were also associated with the cytoplasm (Fig. S4 E), plasma membrane (Fig. S4 F), and nucleus.

To complement the GO analysis of DE genes at genome-wide significance, we also performed a general test of differential expression. Because we observed discordant expression changes within pathways, we used a joint test of up- and down-regulation by calculating the absolute value of the z-score for differential expression of each gene, followed by a t test for genes within versus outside each pathway. Gene sets from pathway databases (canonical pathways) were augmented to include gene lists from the literature (curated pathways). We found eight significant canonical pathways, all related to the cell interface with the extracellular space: focal adhesion, integrins, axon guidance, collagen formation, ECM–receptor interactions, and ECM organization (Fig. 9 A and Table S3). We found 51 significant curated gene sets, 13 of which we characterized as cancer related (Fig. 9 B and Table S3). Out of 183 DE genes, 33 were associated with at least three cancer-related gene sets (Fig. 9 B). We conclude that our data identify a novel set of genes regulated by Twist1 during dissemination that collectively reprogram the extracellular environment and cell interactions with the ECM. Importantly, some of the DE genes have enzymatic activity, are up-regulated in human cancers, and may represent novel targets for inhibiting dissemination (Fig. 9 B and Fig. S5 B).

**E-cad loss blocks single cell dissemination of Twist1* cells**

We observed that Twist1 induced dissemination of cells with membrane-localized E-cad and β-catenin and that Twist1 did not affect E-cad RNA levels. It was therefore possible that E-cad was contributing to Twist1-induced dissemination. Accordingly, we tested whether E-cad KD would inhibit single cell dissemination. We used lentiviral shRNA and puromycin selection to knock down Luciferase (Luc) or E-cad in CMV::rtTA::TRE-Twist1 organoids isolated from the same mouse (Fig. 10 A). We confirmed E-cad loss by immunoblotting (Fig. 10 B) and used Luc KD organoids as a negative control. Consistent with our E-cad deletion experiments, we observed a tandem reduction in αE-catenin and β-catenin.

We next used time-lapse microscopy to quantify dissemination after Twist1 induction in Luc KD and E-cad KD organoids. Surprisingly, E-cad KD resulted in a strong inhibition of single cell dissemination (Fig. 10, C–F; and Video 8). Many Twist1-induced, E-cad KD organoids had no detectable disseminated cells (Fig. 10 E). Instead, concurrent Twist1 expression and E-cad KD induced migration of long chains of cells into the ECM (Fig. 10, G–G′, and Video 9). Twist1-induced, E-cad KD cells retained nuclear Twist1* protein (Fig. 10, H and H′), were cytokeratin* (Fig. 10, I and I′), and were organized in collective groups (Fig. 10, H–I′). We conclude that Twist1 requires E-cad for efficient single cell dissemination.

**Discussion**

Our goal was to define the molecular requirements for dissemination of primary, normal epithelial cells. We used genetic techniques to contrast deletion of the cell adhesion gene E-cad with expression of the EMT transcription factor Twist1. We focused on E-cad as it is frequently mutated in human cancer (Berx et al., 1996; Hirohashi, 1998), and loss of E-cad can synergize with loss of p53 to promote metastasis (Derksen et al., 2006). In our experiments, E-cad deletion throughout the mammary epithelium resulted in loss of most intercellular junctions, loss of simple epithelial architecture, and inhibition of branching morphogenesis. However, E-cad* cells within genetic mosaic epithelium were able to initiate and elongate normal mammary buds in 3D culture and in vivo. The associated E-cad* cells were viable and remained adherent to each other but did not contribute to polarized luminal ducts. Therefore, mammary epithelial cells must be able to maintain contact through alternate adhesion systems such as desmosomal cadherins or protocadherins. We conclude that E-cad is required for normal mammary development but that its loss alone is not sufficient for robust dissemination.

We next evaluated the consequences of Twist1 expression as Twist1 can regulate multiple aspects of metastasis and is thought to function primarily through E-cad repression (Yang et al., 2004; Peinado et al., 2007; Vesuna et al., 2009; Tran et al., 2012; Tsai et al., 2012). Our data reveal that Twist1 expression (TRE-Twist1), cultured these organoids for 24 h in basal medium, added doxycycline for 48 h, and then extracted RNA during active dissemination (Fig. 8 A). RNA-seq analysis identified 183 genes that were differentially expressed between control and Twist1-expressing tissue at genome-wide significance (P = 2.7 × 10−6; Table S1).

Surprisingly, none of the canonical EMT genes were significantly differentially expressed at the RNA level, including E-cad (P = 0.35). However, both Snail (P = 3.2 × 10−6) and Fn1 (P = 4.6 × 10−5) were close to genome-wide significance (Fig. 8 B). We next analyzed the expression of 127 genes involved in cell–cell adhesion and intercellular junctions (Table S2). Of these genes, only five were differentially expressed (Celsr1, Cldn2, Fat4, Frem2, and Pcdh18). No classical cadherins, desmosomal cadherins, catenins, or cytokeratins were significantly dysregulated at the RNA level. We conclude that Twist1 induces dissemination without loss of epithelial identity. This observation is consistent with the positive immunoreactivity for cytokeratin, E-cad, and β-catenin protein in disseminated cells.

We next analyzed gene ontology (GO) slim annotations to characterize biological process (Fig. 8 C), cellular component (Fig. 8 E), and molecular function terms (Fig. S5A) associated with our 183 differentially expressed (DE) genes (Ashburner et al., 2000). Relevant GO biological processes with several DE genes included cell adhesion (Fig. 8 D), transport (Fig. S4 A), cell differentiation (Fig. S4 B), lipid metabolic process (Fig. S4 C), and signal transduction (Fig. S4 D). Of these, the cell adhesion, cell differentiation, and ECM organization GO categories were statistically significantly enriched for DE genes relative to genes overall. Importantly, the DE cell adhesion genes were primarily associated with cell–substrate, not cell–cell, adhesion and with cell migration, cell projections, and ECM organization (Fig. 8 D).

We observed significantly more DE genes than expected in GO cellular component categories for extracellular region, extracellular space, and proteinaceous ECM (Fig. 8 F). DE genes were also associated with the cytoplasm (Fig. S4 E), plasma membrane (Fig. S4 F), and nucleus.

To complement the GO analysis of DE genes at genome-wide significance, we also performed a more general test of differential expression. Because we observed discordant expression changes within pathways, we used a joint test of up- and down-regulation by calculating the absolute value of the z-score for differential expression of each gene, followed by a t test for genes within versus outside each pathway. Gene sets from pathway databases (canonical pathways) were augmented to include gene lists from the literature (curated pathways). We found eight significant canonical pathways, all related to the cell interface with the extracellular space: focal adhesion, integrins, axon guidance, collagen formation, ECM–receptor interactions, and ECM organization (Fig. 9 A and Table S3). We found 51 significant curated gene sets, 13 of which we characterized as cancer related (Fig. 9 B and Table S3). Out of 183 DE genes, 33 were associated with at least three cancer-related gene sets (Fig. 9 B). We conclude that our data identify a novel set of genes regulated by Twist1 during dissemination that collectively reprogram the extracellular environment and cell interactions with the ECM. Importantly, some of the DE genes have enzymatic activity, are up-regulated in human cancers, and may represent novel targets for inhibiting dissemination (Fig. 9 B and Fig. S5 B).
Figure 8. **Twist1 induced changes in genes regulating cell–ECM interactions and the extracellular space.** (A) RNA-seq was used to compare gene expression 48 h after Twist1 induction in control versus Twist1 organoids. (B) Heat map of canonical EMT genes. Only Twist1 was significantly differentially expressed. Genes are sorted by increasing p-value. (C–F) The 183 DE genes were mapped to direct associations with GO Slim biological process (C) and cellular component (E) terms. Black vertical bars indicate the expected number of DE genes per category. Asterisks specify significantly enriched terms. (D) DE genes associated with cell adhesion. (F) DE genes associated with extracellular space, extracellular region, and proteinaceous ECM. Genes are sorted by descending fold change in D and F. BP, biological process; CC, cellular component.
Dissemination without loss of epithelial identity

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However, because our experimental induction of Twist1 was sufficient to induce dissemination, our 183 DE genes are likely particularly important in regulating the transition from adherent to motile epithelial cell phenotypes.

The conceptual framework for EMT was developed in response to classic experiments by Hay and co-workers in which definitive epithelial tissues lost polarity and disseminated as single cells into collagen I gels (Greenburg and Hay, 1982; Hay and Zuk, 1995). The appearance of these cells was most similar to that of embryonic mesenchymal cells, leading to the concept of an EMT. We are observing a similar migration of cells out of an epithelial tissue and into the surrounding ECM. However, we do not observe a loss of epithelial-specific gene expression, and the migratory cells are readily able to reestablish epithelial organization, both spontaneously and after cessation of Twist1 expression. Interestingly, we observed reductions in protein but not RNA levels of E-cad, β-catenin, and αE-catenin, suggesting posttranslational regulation of adherens junction components after Twist1 induction (Reynolds, 2007). However, we demonstrated that complete KD of E-cad dramatically inhibited Twist1-induced single cell dissemination. We speculate that the exact protein levels of E-cad may critically regulate whether Twist1 expression results in single cell dissemination or collective cell migration.

Accordingly, our data support the concept of a Twist1-dependent epithelial migratory program rather than a transition to mesenchymal cell fate or gene expression. Consistent with this framework, Twist1 regulates genes important for interactions with the stromal ECM environment. Our concept of an epithelial migratory program also finds support in breast cancer. Human breast tumors can express E-cad in both the primary tumor and in distant metastases (Kowalski et al., 2003), and primary breast tumor cells positive for the EMT transcription factor Slug can express high levels of E-cad (Côme et al., 2006).

Most cancer therapies target the increased proliferation of cancer cells relative to normal tissues and not the cell behaviors driving invasion, dissemination, and metastasis. Few of these drugs have proven clinical benefit in metastatic breast cancer patients (Carey, 2010). Twist1 is overexpressed in multiple metastatic human cancers and appears to specifically regulate metastatic cell behaviors in multiple experimental cancer models (Yang et al., 2004; Morel et al., 2012; Tran et al., 2012; Tsai et al., 2012). Twist1 therefore represents an attractive conceptual target for developing anti-metastatic therapies. However, it is very difficult to target a transcription factor with small molecule therapeutics. Our observation that Twist1 regulates many proteins in the extracellular compartment suggests that there may be essential, druggable effectors downstream of Twist1 whose repression could inhibit dissemination. Our inducible mouse model revealed that disseminated single cells rapidly cease migration after loss of Twist1 expression, suggesting that interfering with the Twist1 program could be an effective anticancer strategy. We envision our Twist1-induced dissemination assay as a rapid, reproducible, and scalable platform to build a new molecular model for effectors of Twist1 and to identify novel therapeutic compounds to antagonize cancer invasion and dissemination.

is sufficient to induce normal epithelial cells to disseminate out of an epithelium, migrate through the ECM, and establish secondary epithelial sites. Surprisingly, the disseminating cells retained cytokeratin expression, and many displayed membrane-localized E-cad and β-catenin. Transcriptional profiling revealed essentially no changes in the RNA expression of epithelial-specific cadherins, catenins, or keratins. Instead, Twist1 regulated many genes that mediate cell–matrix adhesion or modify the extracellular compartment. Because Twist1 regulates distinct targets depending on its BHLH dimer partner (Barnes and Firulli, 2009), it is likely that its regulated genes vary in different experiments.
from The Jackson Laboratory. Mouse husbandry and procedures were all conducted under an Institutional Animal Care and Use Committee–approved animal protocol.

Materials and methods

Mouse strains

The R26::Cre-ER mouse line (Badea et al., 2003) was a gift from J. Nathans (Johns Hopkins University, Baltimore, MD). The CMV::rtTA transgenic line was a gift from F. Cong and H. Varmus (National Cancer Institute, Bethesda, MD). The Twist1-tetO-luc (TRE-Twist1) transgenic line was previously described (Tran et al., 2012). E-cadfl/fl (Boussadia et al., 2002), mT/mG (Muzumdar et al., 2007), and R26::Lox-Stop-Lox-rtTA-IRES-EGFP (R26::LSL-rtTA; Belteki et al., 2005) mouse lines were acquired from The Jackson Laboratory.

Isolation and 3D culture of primary mammary epithelial organoids

We used mechanical disruption, collagenase/trypsin digestion, and differential centrifugation to purify fragments of primary mammary epithelial ducts, termed organoids, as previously described (Ewald et al., 2008, 2012; Nguyen-Ngoc et al., 2012). In brief, mammary glands were harvested from mice 8–12 wk old, minced with a scalpel, and shaken for 40 min.
at 37°C in collagenase solution: DMEM (10565–018; Gibco) with 2 mg/ml collagenase (C2139; Sigma-Aldrich), 2 mg/ml trypsin (27250–018; Gibco), 5% FBS (F0926; Sigma-Aldrich), 5 μg/ml insulin (I9278; Sigma-Aldrich), and 50 μg/ml gentamicin (15750; Gibco). Suspensions were centrifuged at 1,250 g to remove a floating layer of adipocytes, and pellets were treated with 2 U/μl DNase (D4263; Sigma-Aldrich) to detach organoids from stromal cells. Enzymes and single cells were removed by four quick spins at 1,250 g such that the final pellet consisted mostly of organoids, each containing several hundred cells. Organoids were embedded in 3D Matrigel (354230; BD) at 2–3 organoids/μl and plated as 100-μl suspensions in 24-well coverslip-bottomed plates (662892; Greiner Bio-One) over a 37°C heating block. Gels were allowed to polymerize for 30 min at 37°C and then cultured in organoid medium: DMEM with 1% insulin-transferrin-selenium (1500–056; Gibco) and 1% penicillin-streptomycin (P4333; Sigma-Aldrich). Basal organoid medium was used to induce cyst formation, whereas addition of 2.5 mM FGF2 (FG0291; Sigma-Aldrich) was used to induce branching morphogenesis. Branching was scored as organoids with three or more elongated buds. Cysts were scored as unbranched organoids with lumens detectable by light microscopy. Dissemination was scored as organoids with one or more adjacent single cells that were clearly separated from the epithelial layer.

**Tamoxifen-inducible Cre-mediated deletion in 3D culture**

Cre activity was induced in Cre-ER;E-cad-fl/fl mice by exposing organoids overnight with 50 nM tamoxifen once embedded in Matrigel. To wash out tamoxifen, samples were rinsed with PBS, incubated in organoid medium for 20 min at 37°C, and then cultured in fresh organoid medium. The tamoxifen-inducible system resulted in Cre activity in almost all cells and did not affect branching morphogenesis in control organoids (e.g., Cre-ER;E-cad-2/2; M/G). Percentage of recombination was measured over a 24-h period.

**Adenoviral delivery of Cre recombinase**

Before embedding in Matrigel, mammary organoids were infected with Adeno-Cre (1045; Vector Laboratories) at a ratio of ~10⁷ PFU per 1,000 organoids. Infections were conducted in 50 μM DMEM for 1–2 h at 37°C to yield recombination in 50–75% of cells. Percentage of recombination was raised by increasing viral titer or by overnight incubation with virus.

**Twist1 activation in 3D culture**

Twist1 expression was induced by exposing organoids to 10 µM tamoxifen in organoid medium containing 5 μM doxycycline (Shanghai RenYoung Pharmaceutical Co., Ltd.). Because doxycycline is labile, medium was replaced every 48 h. To turn Twist1 expression off, doxycycline-containing medium was removed and samples were rinsed with sterile PBS. Samples were then incubated with organoid medium without doxycycline for 20 min at 37°C. This medium was then discarded, samples were rinsed again with PBS, and fresh organoid medium without doxycycline was added back.

**Mammary fat pad transplantation**

For transplantation of genetic mosaic E-cad or Twist1 tissue, we isolated organoids and used Cre recombinase in combination with Adeno-Cre, and washed organoids twice with 500 μl DMEM to remove viral particles. For all experiments, organoids were incubated at 37°C overnight in organoid medium with 2.5 mM FGF2 in HydroCell 96-well microplates (174907; Thermo Fisher Scientific). For Twist1 experiments, organoid medium was supplemented with 5 μg/ml doxycycline. The next day, organoids were resuspended in a 50:50 DMEM/50% Matrigel solution at a density of 20–40 organoids/μl and stored on ice. We conducted orthotopic transplantation into 3-wk-old NOD/SCID mice with 200-μm-thick layers of Matrigel. We isolated epithelium from fl/fl; mT/mG littermates, induced recombination with E-cad and Twist1 littermates, induced recombination with Adeno-Cre, and cultured organoids for 5–7 d in Matrigel. Embedded organoids were then fixed in 3% glutaraldehyde to preserve for shipping to Lawrence Berkeley National Laboratory. There, samples were placed in 1-mm-wide by 200-μm-deep aluminum freezing hats and, before freezing, were surrounded with 20% BSA, used as a cryoprotectant. Samples were then cryopreserved using a high-pressure freezer (HPM-010; Bal-Tec, Inc.) and freeze substituted in 1% osmium tetroxide and 0.1% uranyl acetate in acetone with 5% DDD₃O₃, as previously described (McDonald and Webb, 2011). Upon completion of freeze substitution, samples were progressively infiltrated with an epon-Araldite resin (McDonald and Müller-Reichert, 2002). Polyvinyl in epon-Araldite resin was used for glass embedding between two glass slides to allow for precise localization of features of interest (Müller-Reichert et al., 2003).

**TEM**

Samples were sectioned into 70–100-nm-thin and 500-nm-thick sections using an Ultramicrotome (UC6; Leica). Sections were then collected onto formvar-coated, rhodium-enforced copper 2-mm slot grids. The grids were post-stained with 2% uranyl acetate followed by Reynold’s lead citrate, for 5 min each. The sections were then imaged using a Tecnai 12 TEM (FEI), operated at 200 kV. For TEM imaging, images were collected at 20-min intervals for 2–4 d, and temperature was maintained at 37°C and CO₂ at 5%. Imarisa (Bitplane) was used to analyze time-lapse movies, place scale bars, and export individual TIFFs. Photoshop CS5 (Adobe) was used to adjust levels on entire images to maximize image clarity.

**Confocal microscopy**

Confocal imaging was performed on a spinning-disk confocal microscope (Solamere Technology Group Inc.) with an XR/Mega-10 S30 camera (Stanford Photonics, Inc.), as previously described (Ewald et al., 2011; Ewald and Webb, 2013). A Fluor 20x/0.75 objective lens (Carl Zeiss) was used for imaging. Samples were recorded 100–200 positions in parallel for 3–7 d, with images acquired at 20-min intervals. Temperature was maintained at 37°C and CO₂ at 5%. AxioVision (Carl Zeiss) was used to acquire and analyze time-lapse movies, place scale bars, and export individual TIFFs. Photoshop CS5 was used to adjust as needed to adjust levels and gamma for each channel on entire images to maximize image clarity.

**Differential interference contrast (DIC) microscopy**

Time-lapse imaging of mammary organoids was conducted using an LD Plan-Neofluar 20/0.4. Korr Phz objective lens and a Cell Observer system with an AxioObserver Z1 and an AxioCam MRM camera (Carl Zeiss). In addition, samples were recorded 100–200 positions in parallel for 3–7 d, with images acquired at 20-min intervals. Temperature was maintained at 37°C and CO₂ at 5%. AxioVision (Carl Zeiss) was used to acquire and analyze time-lapse movies, place scale bars, and export individual TIFFs. Photoshop CS5 was used to adjust levels on entire images to maximize image clarity.

**High-pressure freezing and freeze substitution processing**

We isolated epithelium from E-cad-fl/fl and E-cad-2/2 littermates, induced recombination with Adeno-Cre, and cultured organoids for 5–7 d in Matrigel. Embedded organoids were then fixed in 3% glutaraldehyde to preserve for shipping to Lawrence Berkeley National Laboratory. There, samples were placed in 1-mm-wide by 200-μm-deep aluminum freezing hats and, before freezing, were surrounded with 20% BSA, used as a cryoprotectant. Samples were then cryopreserved using a high-pressure freezer (HPM-010; Bal-Tec, Inc.) and freeze substituted in 1% osmium tetroxide and 0.1% uranyl acetate in acetone with 5% DDD₃O₃, as previously described (McDonald and Webb, 2011). Upon completion of freeze substitution, samples were progressively infiltrated with an epon-Araldite resin (McDonald and Müller-Reichert, 2002). Polymerization in epon-Araldite resin was performed by flat embedding between two glass slides to allow for precise localization of features of interest (Müller-Reichert et al., 2003).

**Desmosome quantification**

The number of desmosomes and regions used for quantification.

**Image analysis**

Images were acquired using a Hamamatsu 1394 camera mounted to a Nikon Eclipse TE2000-U microscope. Confocal imaging was performed on a spinning-disk confocal microscope (Solamere Technology Group Inc.) with an XR/Mega-10 S30 camera (Stanford Photonics, Inc.), as previously described (Ewald et al., 2011; Ewald and Webb, 2013). A Fluor 20x/0.75 objective lens (Carl Zeiss) was used for imaging. Samples were recorded 100–200 positions in parallel for 3–7 d, with images acquired at 20-min intervals. Temperature was maintained at 37°C and CO₂ at 5%. AxioVision (Carl Zeiss) was used to acquire and analyze time-lapse movies, place scale bars, and export individual TIFFs. Photoshop CS5 was used to adjust as needed to adjust levels and gamma for each channel on entire images to maximize image clarity.

**Fabrication of tissue constructs**

Cryogenic tissue constructs were used to form tissue constructs used to fabricate tissue constructs in the second and third layers deep to the surface, we counted the desmosomes located between cells in the second and third layers deep to the surface, we counted the desmosomes located between cells in the second and third layers deep to the surface.
Immunofluorescence
Organoids grown in 3D Matrigel were fixed in 4% paraformaldehyde for 10 min, rinsed three times in PBS for 10 min, embedded in Optimal Cutting Temperature compound (OCT), and frozen at −80°C. OCT blocks were sectioned at 50-µm thickness by cryostat at −20°C. Sections were placed on Superfrost Plus Gold microscope slides (15-188-48; Fisherbrand) and stored at −80°C. For antibody staining, samples were thawed at room temperature, rinsed twice in PBS for 10 min to remove OCT, permeabilized with 0.5% Triton X-100 for 1 h, and rinsed twice in PBS for 10 min. Samples were blocked for 1–3 h with 10% FBS/1% BSA, incubated with primary antibodies overnight at 4°C in 1% FBS/1% BSA, and rinsed three times in 1% FBS/1% BSA for 15 min. Incubation with secondary antibodies was conducted in 1% FBS/1% BSA overnight at 4°C or for 2 h at room temperature. Slides were rinsed three times in PBS for 10 min, mounted with Fluoromount (F4680; Sigma-Aldrich), and sealed with coverslips. F-Actin was stained with Alexa Fluor Phalloidin (1:100; Invitrogen), and nuclei were stained with DAPI (1:1,000; D3571; Invitrogen). Immunofluorescence staining for each antibody was performed at least three independent times for a minimum of 10–15 organoids. Primary antibodies used were rat anti-E-cad (1:250; 13-1900; Invitrogen), rabbit anti-ZO-1 (1:500; 40-2300; Invitrogen), rabbit anti-β-catenin (1:1,000; C2206; Sigma-Aldrich), mouse anti-e-catenin (1:100; AIX-B04-101; Enzo Life Sciences), rabbit anti-laminin 332 (1:1,000; gifts of P. Marinkovich, Stanford University, Stanford, CA, and M. Aumailley, University of Cologne, Cologne, Germany), rat anti-cytokeratin-8 (1:100; TROMA-I; Developmental Studies Hybridoma Bank), rabbit anti-α-cytokeratin-1 (1:500; PRB-155P; Covance), mouse anti-smooth muscle α-actin (1:250; AS228; Sigma-Aldrich), and mouse anti-twist1 (1:50; sc-81417; Santa Cruz Biotechnology Inc.). Secondary antibodies used were all Alexa Fluor-conjugated antibodies (1:200; Invitrogen).

Transplanted no. 4 mammary glands were dissected, fixed in 4% paraformaldehyde for 4 h at room temperature, rinsed three times in PBS for 15 min, and embedded in OCT. OCT blocks were sectioned at 50–100-µm thickness by cryostat with OCT at −40°C and CT at −30°C. Samples on slides were stained as above but incubated in primary antibody for 48 h at 4°C and in secondary antibody for 24 h at 4°C or 6–8 h at room temperature.

Protein extraction
Lysis buffer for protein extraction was prepared by diluting 10x RIPA buffer (20–188; EMD Millipore) in ultrapure water and chilling the mixture at 4°C for at least 2 h. Immediately before use, lysis buffer was supplemented with 0.1% SDS, 5% glycerol, 3 mM EDTA, 1 mM NaF, 1 mM PMSF, 1.5 mM NaVO₃, Aprotinin (A6279; Sigma-Aldrich), and a mini protease inhibitor tablet (11836153001; Roche). Organoids embedded in 3D Matrigel were collected using freshly made PBS/EDTA buffer (5 mM, 1 mM NaVO₃, 1.5 mM NaF, and 1 mM PMSF in PBS). Medium was aspirated from 3D culture wells, and all wells were rinsed once quickly with 1 ml of cold PBS. Approximately 1 ml of cold PBS/EDTA buffer was used to dissolve two 100-µl gels. Solutions were transferred to centrifuge tubes and mixed well by pipetting. Tubes were left on a 4°C shaker for 1 h to dissolve the Matrigel and then centrifuged at 400 rcf for 5 min at 4°C. Supernatants were removed and, if necessary, pellets were washed with additional PBS/EDTA/EDTA to remove residual Matrigel. After another 5-min spin, pellets were resuspended in 100 µl of RIPA lysis buffer, vortexed, and left on ice for 30–40 min. Tubes were centrifuged for 10 min at 18,400 rcf at 4°C, and supernatants were collected for 3D culture and imaged by DIC time-lapse microscopy. In parallel, TRE-Twist1 (rTa) organoids from littermate controls were treated with lentiviruses and, after selection, used for protein extraction to evaluate E-cad KD efficiency. Time-lapse movies were used to track and count cells that had disseminated by 100 h of Twist1 induction in each of the three treatment groups. A cell was considered disseminated if it had a clear space between itself and the main organoid (visible ECM) and was observed to be migrating away persistently. Cells within collective tissues that temporarily detached in only a few frames were not counted as disseminated.

RNA isolation and sequencing
Organoids were isolated from three CMV::rTa;TRE-Twist1 mice (“Twist1”) and three TRE-Twist1 (rTa); “Control”) littermates (all onbred FVB/N). For each mouse, organoids were embedded in 3D Matrigel at 3–10 organoids/ml and plated as six 50-µl suspensions in a 35-mm dish. Organoids were isolated from three RNA isolation and sequencing CMV::rTa;TRE-Twist1 samples were resuspended in 20 µl of lysis buffer at 4°C and centrifuged at 12,000 g for 5 min. RNA was extracted using TRIzol (15596-026; Life Technologies) and RNeasy (Qiagen). With 10–100 ng of RNA collected per sample, we generated barcoded NuGen RNA-seq v2 libraries and ran paired-end, 75-bp, 50-cycle sequenced (HiSeq 2000; Johns Hopkins Medical Institutions Deep Sequencing and Microarray Core Facility). Paired-end, non-strand-specific RNA-seq reads were mapped to the mouse reference genome (Genomic Reference Consortium build 38) using read mapper Bowtie (Langmead et al., 2009) and splice junction mapper TopHat (Trapnell et al., 2009). We achieved a mean of 51.4 million uniquely mapped reads per sample and estimated the number of reads mapped to each gene using HTSeq (Anders, 2010), with gene coordinates from the reference genome Generic Feature Format file. Raw counts were normalized and p-values were calculated for Twist1 versus control differential expression from negative binomial distributions using DESeq (Anders and Huber, 2010). Based on the number of genes tested, the genome-wide significance level for 0.05 family-wise error rate was 2.74 × 10⁻⁶, and 183 genes were significant at this level. Sequence data has been uploaded to the Sequence Read Archive (project accession no. SRP033275).
GO analysis
Significant genes were characterized using GO Slim categories for biological process, cellular component, and molecular function (Ashburner et al., 2000; GO file downloaded on 9/12/13 and mapping from 3/5/13). We first computed the overall fraction of genes that were up-regulated ($f_{up} = 107/18,260$) and down-regulated ($f_{down} = 76/18,260$). For each GO term, we then calculated the number of genes $n$ annotated to the category and performed the 183 DE genes segment analysis. For this analysis, we did not consider further. For categories with $n > 2$, we conducted separate two-sided tests corresponding to enrichment or depletion based on Poisson distributions for up-regulated (expected number = $f_{up}n$) and down-regulated (expected number = $f_{down}n$) DE genes. For each of the three ontologies, the p-value threshold for 0.05 family-wise error rate was set to 0.05/4 (x number of categories tested). This conservative approach was selected as more computationally convenient than the corresponding Fisher's exact tests.

Pathway-level differential expression
To assess pathway-level differential expression, we used gene sets available through MSigDB v4.0 (Subramanian et al., 2005). The 1,320 canonical gene sets within MSigDB are aggregated from major pathway databases: BioCarta, KEGG, Reactome, the Pathway Interaction Database, the Sigma-Aldrich database, the Signaling Gateway database, the Signal Transduction KE database, and the SuperArray database. These are then augmented to 4,722 curated gene sets by including curated gene lists from published studies, the L2L gene sets from mammalian microarray studies (Newman and Weiner, 2005), the MYC Target Gene Database (Zeller et al., 2003), and other public resources. For each gene, including genes not differentially expressed at genome-wide significance, the p-value from DESeq was converted to the equivalent z-score for a two-sided test, with $z > 0$ for Twist1 > Control and $z < 0$ for Twist1 < Control. Each pathway was tested using a one-sided Student's t-test of $|z|$ within pathway groups to identify significantly over- or under-represented numbers of DE genes without regard to direction (up or down in Twist1 versus Control). Significance thresholds for 0.05 family-wise error rates at the pathway level were estimated using 500 permutations of gene z-scores, recording the best p-value from each permutation, and taking the 25th-best p-value as the 0.05 FWER threshold. The resulting thresholds were $1.44 \times 10^{-13}$ for canonical gene sets and $5.31 \times 10^{-10}$ for curated gene sets. These thresholds are more stringent than a standard Bonferroni correction.

Online supplemental material
Fig. 5 shows that Twist1 induction is cell autonomous and occurs in vivo. Fig. S3 shows that Twist1 expression results in reduced levels of adherens junction proteins. Fig. S4 shows heat maps of genes directly associated with relevant GO Slim biological process and cellular component terms. Fig. S5 shows GOSlim molecular function terms. Fig. 6 shows a heat map of Twist1-regulated genes with enzymatic activity. Video 1 shows confocal time-lapse imaging of a CreER;Ecad\textsuperscript{tm/m}G
organoid transitioning from single to multilayered organoids after E-cad deletion. Video 2 DIC time-lapse imaging of representative Ecad\textsuperscript{tm/m}G-organoids in the branching morphogenesis assay. Video 3 shows confocal time-lapse imaging of E-cad\textsuperscript{tm/m}G organoid. Videos 4 and 5 show DIC time-lapse imaging of representative CMV::rtTA;TRE-Twist1 organoids grown in basal medium and FGF2-containing medium, respectively, with and without Twist1 induction. Videos 6 and 7 show DIC time-lapse imaging of a CMV::rtTA;TRE-Twist1 organoid grown in basal medium and FGF2-containing medium, respectively, with and without Twist1 induction. Videos 6 and 7 show DIC time-lapse imaging of a CMV::rtTA;TRE-Twist1 organoid grown in basal medium and FGF2-containing medium, respectively, with transient Twist1 induction. Video 8 shows DIC time-lapse imaging of representative Twist1-expressing organoids with Luc and E-cad KD. Video 9 shows DIC time-lapse imaging of collective epithelial migration in a Twist1-expressing, E-cad KD organoid. Table S1 lists all genes sequenced by RNA-seq, sorted by p-value, and the 183 DE genes along with their associated GO Slim terms. Table S2 lists RNA-seq raw counts and p-values for 127 epithelial genes involved in cell-cell adhesion and intercellular junctions. Table S3 lists the significant canonical pathways and curated gene sets identified by gene set enrichment analysis. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201306088/DC1.

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