Rebalancing of actomyosin contractility enables mammary tumor formation upon loss of E-cadherin

Koen Schipper¹, Danielle Seinstra¹, Anne Paulien Drenth¹, Eline van der Burg¹, Veronika Ramovs², Arnoud Sonnenberg², Jacco van Rheenen¹, Micha Nethe¹,³ & Jos Jonkers¹

E-cadherin (CDH1) is a master regulator of epithelial cell adherence junctions and a well-established tumor suppressor in Invasive Lobular Carcinoma (ILC). Intriguingly, somatic inactivation of E-cadherin alone in mouse mammary epithelial cells (MMECs) is insufficient to induce tumor formation. Here we show that E-cadherin loss induces extrusion of luminal MMECs to the basal lamina. Remarkably, E-cadherin-deficient MMECs can breach the basal lamina but do not disseminate into the surrounding fat pad. Basal lamina components laminin and collagen IV supported adhesion and survival of E-cadherin-deficient MMECs while collagen I, the principle component of the mammary stromal micro-environment did not. We uncovered that relaxation of actomyosin contractility mediates adhesion and survival of E-cadherin-deficient MMECs on collagen I, thereby allowing ILC development. Together, these findings unmask the direct consequences of E-cadherin inactivation in the mammary gland and identify aberrant actomyosin contractility as a critical barrier to ILC formation.

¹ Division of Molecular Pathology, Oncoide Institute, The Netherlands Cancer Institute, Amsterdam, The Netherlands.
² Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands.
³ Present address: Department of Hematopoiesis, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, Amsterdam, The Netherlands. Correspondence and requests for materials should be addressed to M.N. (email: m.nethe@sanquin.nl) or to J.J. (email: jjonkers@nki.nl)
The maintenance of epithelial cell–cell adhesion sustains organ function and prevents tissue infection and tumor development. E-cadherin (CDH1) is a transmembrane protein and a master regulator of the cell adherence junctions controlling cell–cell adhesion. E-cadherin is also a well-established tumor suppressor which is in general viewed to prevent tumor cell dissemination1–3. Complete loss of E-cadherin expression is a hallmark of invasive lobular carcinoma (ILC)4. ILC comprises 8–14% of all breast cancer patients, making it the 2nd most diagnosed breast cancer type worldwide2,5,6. Histologically, ILC is characterized by non-cohesive cells arranged in single files infiltrating the surrounding fibrous collagen-rich stroma7. The infiltrative nature of ILC often complicates surgical removal. ILCs also typically respond poorly to classical regimes of chemotherapy. To gain insight into the mechanisms underlying ILC development, we have previously developed genetically engineered mouse models that, closely resemble human ILC2,5,9. Mammary gland-specific inactivation of E-cadherin in combination with loss of p53 or PTEN markedly enhanced mammary tumor development thus confirming the tumor suppressive role of E-cadherin4,5,9,10. Intriguingly, somatic inactivation of E-cadherin by itself is not sufficient to induce mammary tumor formation, but rather seems to hamper survival of mouse mammary epithelial cells (MMECs)4,8,10. In addition, genetic inactivation of E-cadherin in MMEC lines or mouse mammary epithelial organoids does not induce single cell dissociation nor epithelial-mesenchymal transition (EMT)11,12. It remains unclear why loss of E-cadherin by itself hampers cell survival and how this limitation is circumvented during ILC development.

Here we studied the direct consequence of E-cadherin loss in MMECs by genetic labeling and tracing of E-cadherin-deficient MMECs in vivo. We found that E-cadherin-deficient MMECs extrude into the fibrous stroma where they form dynamic but non-tumorigenic cell clusters that only progress to ILC upon partial relaxation of actomyosin. These findings show that actomyosin levels are critical in ILC development.

Results
E-cadherin loss drives cell extrusion to the basal lamina. To monitor the fate of E-cadherin-deficient MMECs in vivo, we generated Wcre;Cdhd1F/F;mTmG mice in which mammary gland-specific expression of Cre from the Wap gene promoter (Wcre) induces somatic inactivation of Cdhd1 (encoding E-cadherin) and GFP expression by recombination of the mTmG reporter in MMECs13,14 (Fig. 1a). Cre recombinase activity, marked by GFP expression, was exclusively detected in cytokeratin-8 (CK8)-positive luminal MMECs (Fig. 1b, c). Strikingly, immunofluorescence (IF) analysis of GFP expression in 6-week-old Wcre;Cdhd1F/F;mTmG mice revealed that E-cadherin-deficient luminal MMECs massively extruded towards the basal lamina and typically resided between the layer of cytokeratin-14 (CK14)-positive myoepithelial cells and the basal stroma (Fig. 1d). Loss of functional E-cadherin in MMECs was also confirmed by dissociation of both β-catenin and p120-catenin from the peripheral membrane due to disruption of the E-cadherin–catenin complex as previously described (Supplementary Fig. 1a, b)12,15. Whereas most extruded luminal MMECs were detected at the basal laminar border, apoptotic E-cadherin-deficient MMECs were sporadically detected in the lumen of the mammary ducts as observed previously8,10. To monitor the fate of extruded E-cadherin-deficient MMECs, we compared mammary gland sections of 3-, 5-, and 12-month-old Wcre;Cdhd1F/F;mTmG mice (n = 3) to age-matched Wcre;mTmG mice by immunohistochemistry (IHC) (Fig. 1e). Interestingly, extruded GFP-marked E-cadherin-deficient MMECs in mammary glands of Wcre;Cdhd1F/F;mTmG mice accumulated as small clusters of cells in the fibrous surrounding stroma. IF analysis confirmed lack of E-cadherin expression in extruded GFP-positive MMECs (Fig. 1f). Moreover, the extruded MMECs represented the majority of GFP-marked E-cadherin-deficient MMECs in Wcre;Cdhd1F/F;mTmG mice, whereas no extrusion of GFP-positive control MMECs was observed in Wcre;mTmG mice (Fig. 1e, g). The clusters of extruded cells did not increase in size over time, which is in line with our previous observation that loss of E-cadherin by itself does not induce mammary tumor formation in mice (Fig. 1h)8. Finally, we did not detect any MMECs in the lumen of mammary glands at these time points, supporting previous findings that E-cadherin-deficient MMECs that extrude into the lumen of the mammary gland undergo apoptosis and are rapidly cleared8,10.

E-cadherin loss in MMECs increases actomyosin contractility. To gain more insight into the behavior of the extruded E-cadherin-deficient MMECs in the basal mammary stroma in situ, we analyzed mammary glands of 3-month old Wcre;Cdhd1F/F; mTmG mice and Wcre;mTmG control mice (n = 3) by live intravital microscopy. The E-cadherin-deficient cellular clusters in Wcre;Cdhd1F/F;mTmG mice were present alongside the entire mammary ductal tree (visualized by mTomato) and faced the surrounding mammary stroma (Fig. 2a). GFP-marked extruded E-cadherin-deficient MMECs formed tight but highly dynamic clusters of motile cells which appeared to constantly tumble around each other (Fig. 2b, Supplementary Movies 1–3). Despite their enhanced motility within these clusters, E-cadherin-deficient cells did not disseminate into the surrounding mammary stroma. Interestingly, extruded MMECs in mammary glands of Wcre;Cdhd1F/F;mTmG mice were marked by extensive membrane blebbing (Fig. 2c, d). Membrane blebbing is often seen in amoeboid migration16 and apoptosis17. However we could not observe any defined form of cell movement or cell death during the time of imaging. We also did not find any cleaved caspase-3-positive apoptotic cells at the basal stromal compartment18. Since membrane blebbing typically results from elevated actomyosin contractility, we next examined myosin light chain (MLC) phosphorylation by IF imaging in mammary gland sections of Wcre;mTmG and Wcre;Cdhd1F/F;mTmG mice (Fig. 2e). In normal mammary glands, luminal epithelial cells have relatively low MLC phosphorylation levels compared to myoepithelial cells. E-cadherin-deficient MMECs in the fibrous stroma showed a clear increase in pMLC staining, confirming an increase in actomyosin contractility (Fig. 2e, f, Supplementary Fig. 2a). Overall these results reveal that E-cadherin-deficient MMECs that persist in the fibrous mammary stroma exhibit an increase in actomyosin contractility.

Actomyosin relaxation enables survival upon E-cadherin loss. To study the consequences of E-cadherin loss in more detail, we isolated mammary epithelium from Wcre;Cdhd1F/F;mTmG mice and Wcre;mTmG mice. Initial attempts to culture Wcre;Cdhd1F/F; mTmG MMECs failed as GFP-positive MMECs detached during formation of epithelial islands and were consequently lost during cell culture (Fig. 3a, Supplementary Movie 4). Since extensive cell blebbing preceded cell detachment, we hypothesized that increased actomyosin contractility, as observed in situ, may hamper adhesion of Wcre;Cdhd1F/F;mTmG MMECs. To test this hypothesis we cultured Wcre;Cdhd1F/F;mTmG MMECs with a ROCK inhibitor (Y-27632), which rescued their adhesion and expansion (Fig. 3a). The obtained polyclonal MMEC population, harboring both GFP-positive and GFP-negative MMECs, had decreased E-cadherin expression indicating the presence of E-cadherin-negative cells (Fig. 3b). Next, we derived E-cadherin-
proficient and -deficient subclones by picking GFP-positive MMECs in the presence of Y-27632 from Wcre;mTmG and Wcre;Cdh1F/F;mTmG MMECs (Fig. 3c). This allowed us to examine cell adhesion of cultured E-cadherin-deficient Wcre; Cdh1F/F;mTmG MMEC subclones in the presence and absence of Y-27632 (10 µM). All E-cadherin-deficient MMEC subclones displayed extensive cell blebbing during cell adhesion (Fig. 3d). In the absence of Y-27632, E-cadherin-deficient MMECs showed reduced survival signaling and increased expression of cleaved caspase-3 indicating the initiation of apoptosis (Fig. 3d). Inhibition of ROCK activity by Y-27632 rescued cell spreading and survival of Wcre;Cdh1F/F;mTmG MMECs (Fig. 3d). To reduce actomyosin contractility downstream of ROCK we next used Blebbistatin to directly reduce myosin activity. Like ROCK
inhibition by Y-27632, direct inhibition of myosin activity by blebbistatin (10 µM) readily rescued cell adhesion of E-cadherin-deficient WrecCdhl1F/F; mTmG MMECs (Fig. 3d). E-cadherin-proficient WrecEmTmG MMEC subclones did not require Y-27632 or blebbistatin to induce cell spreading but nevertheless displayed enhanced cell adhesion and survival upon inhibiting actomyosin contractility (Fig. 3d, f). Remarkably, once cell adhesion was restored, WrecCdhl1F/F; mTmG MMECs grew significantly faster than WrecEmTmG MMECs, likely due to loss of cell contact inhibition as previously reported to be controlled by E-cadherin signaling (Fig. 3f, g)\(^1\)\(^8\),\(^1\)\(^9\). To substantiate our findings, we derived polyclonal cell lines from wild-type (WT) MMECs and Cdhl1ΔΔ MMECs, which were produced by exposing Cdhl1F/F MMECs to a Cre-encoding adenovirus (Fig. 3h). The Cdhl1ΔΔ MMEC line showed a similar dependency on actomyosin relaxation as the WrecCdhl1F/F; mTmG MMEC subclones (Fig. 3i, j). Previously, E-cadherin has been shown to restrict RhoA activity by controlling both p190RhoGAP and p120-catenin signaling\(^2\)\(^0\)–\(^2\)\(^2\). To determine if RhoA activity was also increased in E-cadherin-deficient MMECs during cell adhesion, we performed a RhoA activation pull-down assay. Indeed, Cdhl1ΔΔ MMECs had increased levels of GTP-bound RhoA which coincided with increased expression of total RhoA compared to WT MMECs (Fig. 3k, i, Supplementary Fig. 3a). Cdhl1ΔΔ MMECs also showed increased MLC expression and phosphorylation, confirming an overall increase in actomyosin contractility upon cell adhesion compared to WT MMECs (Fig. 3k, l, Supplementary Fig. 3a–c). Together these results show that E-cadherin-deficient MMECs have aberrant actomyosin contractility during cell adhesion which needs to be reduced in order to allow their adhesion and survival.

**Balanced actomyosin contractility is essential for survival.** Whereas E-cadherin-deficient MMECs require actomyosin relaxation in order to sustain cell adhesion and survival, progression of cell division requires actomyosin contraction\(^2\)\(^3\),\(^2\)\(^4\). We therefore hypothesized that too much actomyosin relaxation would hamper cell survival of E-cadherin-deficient MMECs. To examine this in more detail, we analyzed survival of E-cadherin-deficient MMECs subjected to increasing levels of actomyosin relaxation. We performed colony formation assays using concentration ranges of Blebbistatin (Fig. 4a, b) or the ROCK inhibitor H1152 (ref. \(^2\)\(^5\)) (Fig. 4c, d). H1152 inhibits ROCK with increased specificity and potency compared to Y-27632. Both inhibitors readily rescued survival of E-cadherin-deficient MMECs at low concentrations, i.e. 0.5 µM for Blebbistatin and 0.1 µM for H1152 (Fig. 4a–d). Optimal concentrations permitting survival of E-cadherin-deficient MMECs were found at 5 µM for Blebbistatin and 1 µM for H1152. Importantly, higher concentrations of both compounds hampered survival of E-cadherin-deficient MMECs. Exposure of E-cadherin-deficient MMECs to various concentrations of H1152 reveals a dose-dependent effect on MLC phosphorylation (Supplementary Fig. 4). The optimal concentration of H1152 only partially inhibited MLC phosphorylation indicating that too much relaxation is not tolerated. Altogether these results underscore the requirement of a critical balance in actomyosin contractility that mediates adhesion but does not hamper survival of E-cadherin-deficient MMECs.

**MYPT1 drives actomyosin relaxation and cell adhesion.** We recently found that protein-truncating mutations in Ppp1r12a (encoding MYPT1) drive ILC formation in mice\(^2\)\(^6\). MYPT1 regulates actomyosin contractility by acting as the regulatory subunit of the holoenzyme myosin light chain phosphatase MLCP which inhibits myosin light chain (MLC) activity\(^2\)\(^7\). Truncated MYPT1 lacks the PLK1 and LATS1 interaction domains involved in regulation of mitosis and retains the functional domains to bind PP1 and target pMLC\(^2\)\(^8\)–\(^3\)\(^0\). Truncated MYPT1 also lacks the two inhibitory phosphorylation sites (T696 and T853) that are targeted by ROCK and cause auto-inhibition of MYPT1 (ref. \(^2\)\(^9\)). Therefore, we wondered if truncated MYPT1 could inhibit actomyosin contractility and thereby rescue adhesion of WrecCdhl1F/F; mTmG MMECs upon Y-27632 withdrawal. To test this hypothesis, we transduced the E-cadherin-deficient WrecCdhl1F/F; mTmG MMEC subclones with a lentivirus encoding doxycycline (dox)-inducible, Flag-tagged truncated MYPT1 (t-MYPT1) (Fig. 5a). Protein expression of t-MYPT1 was clearly detected 24 h after adding dox and lost 48 h after dox washout (Fig. 5b). Loss of t-MYPT1 significantly increased MLC activity, reflected by increased pMLC IF staining along the actin stress fibre network that t-MYPT1 reduces actomyosin contractility (Fig. 5c, d). In accordance with exposure to optimal concentrations of H1152 expression of tMYPT1 does not completely inhibit MLC phosphorylation in E-cadherin-deficient MMECs. Accordingly, the expression of t-MYPT1 also rescued cell adhesion and survival of WrecCdhl1F/F; mTmG MMECs in the absence of Y-27632 (Fig. 5e, f). We further hypothesized that the absence of the ROCK-controlled inhibitory phosphorylation sites would increase the potency of t-MYPT1 as an inhibitor of actomyosin contractility (Fig. 5g). Indeed, overexpression of full-length MYPT1 had a relatively modest effect on MLC phosphorylation and cell adhesion compared to t-MYPT1 (Fig. 5h–i, Supplementary Fig. 4). To reduce actomyosin contractility, MYPT1 encompasses a N-terminal region containing a protein phosphatase 1 (PP1) recognition motif and seven Ankyrin repeats (ANK) that facilitate PP1 binding, all of which are retained in t-MYPT1\(^2\)\(^7\),\(^3\)\(^1\),\(^3\)\(^2\). PP1 acts as the catalytic subunit of MLCP and reduces actomyosin contractility by dephosphorylating Serine 19 of MLC\(^3\)\(^2\). Since the
PP1 recognition motif of MYPT1 has been shown to drive PP1 activity upon binding of PP1 to MYPT1 (Fig. 5g). We generated a t-MYPT1 variant lacking the PP1 recognition motif (t-MYPT1ΔPP1) (Fig. 5g). Stable expression of t-MYPT1 and t-MYPT1ΔPP1 showed that loss of the PP1 recognition domain impaired dephosphorylation of pMLC in Wcre;Cdh1F/F;mTomG MMECs (Fig. 5h, i). Expression of t-MYPT1ΔPP1 also failed to rescue cell adhesion and survival of Wcre;Cdh1F/F;mTomG MMECs in clonogenic survival assays (Fig. 5j, k, Supplementary Fig. 5). Overall, these data demonstrate that t-MYPT1 rescues cell adhesion and survival of Wcre;Cdh1F/F;mTomG MMECs by PP1-mediated relaxation of actomyosin contractility.

Fig. 2 E-cadherin loss increases actomyosin contractility. a Still images derived from in vivo intravital imaging of the mammary gland of 8-week-old Wcre;mTomG and Wcre;Cdh1F/F;mTomG mice displaying GFP-positive Cre-switched MMECs and mTomato non-switched MMECs and stromal cells. Zooms reveal motile GFP-positive E-cadherin inactivated MMECs in Wcre;Cdh1F/F;mTomG mice. Scale bars, 20 μm. b Quantification of the percentage of GFP-positive motile cells among Wcre;mTomG (n = 2) and Wcre;Cdh1F/F;mTomG (n = 3) mice. Data are of four and three images per group, respectively. c Still image of in vivo intravital imaging of Wcre;Cdh1F/F;mTomG mice demonstrates extensive cell blebbing of GFP-positive E-cadherin inactivated MMECs. Scale bar, 10 μm. d Quantification of the percentage of GFP-positive blebbing cells among Wcre;mTomG (n = 2) and Wcre;Cdh1F/F;mTomG (n = 3) mice. Data are of four and three images per group, respectively. e Examination of myosin light chain (MLC) activity by IF analysis of pSer19 MLC in GFP-positive E-cadherin-deficient MMECs in 5-month-old Wcre;mTomG and Wcre;Cdh1F/F;mTomG mice. Scale bar, 20 μm. f Quantification of the amount of GFP+ pSer19 MLChigh cells Wcre;mTomG (n = 2) and Wcre;Cdh1F/F;mTomG (n = 3) mice. Data are of 51 and 60 images, respectively. All data are depicted as mean ± standard deviation. All p values were calculated using an unpaired two tailed t-test. Source data are provided as a Source Data file.
Collagen I does not support adhesion upon loss of E-cadherin. Since loss of E-cadherin impaired cell adhesion of primary MMECs in vitro, we next examined the impact of the extracellular matrix surrounding the E-cadherin-deficient MMECs. Upon Cre expression in 6-week-old \( Wcre;Cdh1^{F/F};mTmG \) mice, GFP-marked E-cadherin-deficient MMECs extruded towards the basal lamina (Fig. 6a, zoom). These findings are in line with the observed extruded clusters of E-cadherin-deficient MMECs in the surrounding mammary fibrous stroma which we started to observe in 12 weeks and older \( Wcre;Cdh1^{F/F};mTmG \) mice (Fig. 1e, f). Remarkably, these clusters in the mammary fibrous stroma are surrounded by laminin (Fig. 6a). We therefore next examined cell...
adhesion and survival of E-cadherin-deficient MMECs on laminin-332, a component of the mammary basal lamina. Laminin-332-rich matrix derived from Rac11p cells readily supported cell adhesion and survival of Wcre;Cdhi/F−/mTmG MMECs in the absence of Y-27632 or Blebbistatin and therefore likely supports survival of E-cadherin-deficient MMECs in the fibrous stroma (Fig. 5b, c). In comparison to laminin-332, collagen IV, another component of the basal lamina, induced a more modest increase in survival of E-cadherin-deficient MMECs (Supplementary Fig. 6). Yet, the mammary fibrous stroma is mainly characterized by the deposition of collagen I.13 The clusters of E-cadherin-deficient MMECs, marked by GFP, in the mammary glands of Wcre;Cdhi/F−/mTmG mice were indeed surrounded by fibrous collagen (Fig. 6d, Supplementary Fig. 7). We therefore compared cell adhesion and survival of E-cadherin-deficient MMECs on laminin-332 with collagen I (Fig. 6e, f). In contrast to laminin-332, collagen I did not support adhesion and survival of E-cadherin-deficient MMECs despite expression of the collagen-receptor integrin α2β1 (Fig. 6e, f, Supplementary Fig. 8). Contrary to laminin-332, adhesion to collagen I has been reported to result in increased RhoA activity.34,35 Indeed, a RhoA activation assay confirmed that Cdhi/F−/mTmG MMECs on laminin-332 matrix have significantly decreased RhoA activity compared to cells on collagen I during cell adhesion (Fig. 6g–i). In line with these findings, survival of E-cadherin-deficient MMECs on collagen I could be rescued by expression of t-MYPT1 but not by t-MYP1APP1, which is unable to reduce actomyosin contractility (Fig. 6j, k). Overall these results highlight that collagen I impairs cell adhesion and survival of E-cadherin-deficient MMECs, indicating that the collagen-rich mammary stroma may play an important role in

Fig. 3 Actomyosin relaxation enables survival upon E-cadherin loss. b Western blot analysis of subclones derived from Wcre;Cdhi/F−/mTmG female mice. Scale bar, 20 μm. c Western blot analysis of subclones derived from Wcre;Cdhi/F−/mTmG MMECs stained for E-cadherin and tubulin (loading control). d Brightfield images of MMECs 3 h post seeding in the absence or presence of 10 μM Y-27632 or 10 μM blebbistatin. Scale bar, 20 μm. e Western blot analysis of pSer373 Akt, Akt, cleaved caspase-3, and actin (loading control) in Wcre;Cdhi/F−/mTmG subclones grown in the absence or presence of 10 μM Y-27632. f, g Representative images (f) and quantification (g) of clonogenic assays with Wcre;Cdhi/F−/mTmG (circles) and Wcre; Cdhi/F−/mTmG (squares) subclones grown in the presence or absence of 10 μM Y-27632 or 10 μM blebbistatin (7 days after seeding the cells). Data are of three independent experiments with three clones per experiment. h Western blot analysis of WT FVB and Cdhi+/− MMECs stained for E-cadherin and actin (loading control). i, j Representative images (i) and quantification (j) of clonogenic assays with Cdhi+/− and WT control MMECs with or without 10 μM Y-27632. Data are of three independent experiments. k Representative images of immunoblots of active RhoA pull-down assays and ser19 myosin light chain phosphorylation (pMLC) from Cdhi+/− and WT control MMECs harvested 3 h post seeding. l Quantification of active RhoA pull-down assays by densitometry normalized to the actin loading control. Data are of three independent experiments. All data are depicted as mean ± standard deviation. All p values were calculated using an unpaired two tailed t test. Source data are provided as a Source Data file.

Fig. 4 Balanced actomyosin contractility is essential for survival. a, b Representative images (a) and quantification (b) of clonogenic assays with Wcre; Cdhi/F−/mTmG MMECs cultured with different concentrations of blebbistatin. Data are of three independent experiments with three clones per experiment. c, d Representative images (c) and quantification (d) of clonogenic assays with Wcre;Cdhi/F−/mTmG MMECs cultured with different concentrations of H1152. Data are of three independent experiments with three clones per experiment. All data are depicted as mean ± standard deviation. Source data are provided as a Source Data file.
preventing extruded E-cadherin-deficient MMECs from developing into an ILC.

**Relaxation of actomyosin contractility drives ILC formation.**
As actomyosin relaxation rescued survival of E-cadherin-deficient MMECs on collagen I, it might also enable the development of ILC, which is typically marked by the massive accumulation of fibrous collagen. We therefore examined whether the relaxation of actomyosin contractility could drive malignant transformation of E-cadherin-deficient MMECs in vivo and promote ILC formation in mice. We performed intraductal injections with lentiviruses encoding MYPT1, t-MYPT1, t-MYPTΔPP1, and empty control in mammary glands of Wcre;Cdh1F/F mice and determined tumor burden 20 weeks post-injection (Fig. 7a). As seen before, expression of t-MYPT1 consistently induced formation of classic mouse ILCs characterized by the lack of E-cadherin expression and an Indian file growth pattern in tumor-associated stroma (Fig. 7b, c). Expression of full-length MYPT1 also induced ILC formation, although tumors formed less frequently.
Fig. 5 MYPT1 drives actomyosin relaxation and cell adhesion. a Schematic overview of the doxycycline (dox) inducible t-MYPT1 (MYPT1Δ413) construct. b Western blot analysis of WcreCdhh1F/F;mTmG MMECs transduced with dox-inducible Flag-tagged t-MYPT1 in the absence, presence, or following washout of 2 mg/mL dox as stained for FLAG and actin (loading control). c, d c, d IF staining (c) and quantification (d) of dox-inducible Flag-tagged t-MYPT1 WcreCdhh1F/F;mTmG MMECs cultured in the presence of 2 μg/mL dox or 50 h post washout stained for phospho myosin light chain (pMLC), actin, and Hoechst. Data are of 15 images per condition. e, f Representative images (e) and quantification (f) of clonogenic assays with WcreCdhh1F/F;mTmG MMECs expressing Dox-inducible Flag-tagged t-MYPT1 cultured with or without 2 mg/mL dox and/or 10 μM Y-27632. Data are of three independent experiments with three clones per experiment. g Schematic overview of full-length MYPT1, t-MYPT1, and t-MYPT1ΔPPI (MYPT1Δ413) displaying the PPI recognition motif and the ankyrin repeats (ANK) domain, the leucine zipper domain and inhibitory phosphorylation sites (T696 and T853). h, i I staining (h) and quantification (i) of WcreCdhh1F/F;mTmG MMECs transduced with MYPT1-wt, t-MYPT1, t-MYPT1ΔPPI, or GFP 48 h post washout of 10 μM Y-27632 and stained for pMLC, actin, and Hoechst. Data are of 15 images per condition. j, k Representative images (j) and quantification (k) of clonogenic assays with WcreCdhh1F/F;mTmG MMECs transduced with t-MYPT1, t-MYPT1ΔPPI or GFP cultured in the presence or absence of 10 μM Y-27632 (7 days after seeding the cells). Data are of three independent experiments with three clones per experiment. All data are depicted as mean ± standard deviation. All p values were calculated using an unpaired two tailed t-test. Source data are provided as a Source Data file.

and were on average smaller in size. These results are in line with our previous observations that full-length MYPT1 displays reduced actomyosin relaxation activity compared to t-MYPT1 (Fig. 5). Importantly, expression of t-MYPTAP1, which is unable to induce actomyosin relaxation, failed to induce ILC formation in WcreCdhh1F/F mice (Fig. 7b). Altogether, these findings demonstrate that PPI-driven relaxation of actomyosin contractility by t-MYPT1 supports survival of E-cadherin-deficient MMECs in the collagen-rich mammary fibrous stroma, resulting in ILC development.

Discussion

In this study we uncovered the direct consequences of E-cadherin inactivation in MMECs in vivo. We identified that MMECs upon loss of E-cadherin not only extrude to the lumen as previously reported but also towards the basal lamina. In contrast to cells that extrude towards the lumen, E-cadherin-deficient MMECs that extrude to the basal lamina do not undergo apoptosis. While extruded E-cadherin-deficient MMECs persist outside the basal lamina they do not form mammary tumors. However, because these cells do persist for a prolonged period of time they have the opportunity to gain additional mutations that allow tumor initiation. The accumulation and persistence of E-cadherin-deficient MMECs in the fibrous stroma might therefore explain the increased susceptibility of CDH1 mutation carriers to develop ILC36-38.

Increased actomyosin contractility is generally considered to induce tumor cell invasion and migration, thereby promoting progression of established tumors towards metastatic disease. In the present study, we demonstrate that actomyosin relaxation promotes ILC development by enhancing adhesion and survival of E-cadherin-deficient MMECs. However, complete inhibition of actomyosin contractility in E-cadherin-deficient MMECs is not tolerated, indicating that there is a fine balance between too much contractility and too little. Taken together these findings indicate that the role of actomyosin contractility during tumorigenesis is context-dependent and may change depending on the microenvironment, tumor type, and tumor stage.

Extrusion of E-cadherin-deficient MMECs into the mammary fibrous stroma coincides with substantial changes in the extra-cellular matrix. Importantly, different ECM components have differential effects on the survival of E-cadherin-deficient MMECs. Survival of these cells is promoted by the basal lamina components, laminin-332 and collagen IV, but not by collagen I, which is the principle component of the fibrous stroma. Survival of E-cadherin-deficient MMECs on a collagen I matrix is critically dependent on actomyosin relaxation. This dependency can be explained by the differential effects of laminin-332 and collagen I on Rhoa activity. It has been demonstrated that adhesion of integrin α3β1 to laminin-332 results in increased RAC1 activity, which indirectly inhibits Rhoa via multiple mechanisms including activation of PAK and p190RhoGAP. In contrast, adhesion of integrin α2β1 to collagen I has been shown to increase RhoA signaling. Hence, the presence of fibrillar collagen might prevent spreading and proliferation of the extruded E-cadherin-deficient MMECs.

It is however well established that collagen I promotes invasion and migration of established tumor cells. Moreover, the presence of large amounts fibrillar collagen is a hallmark of ILCs. It is therefore conceivable that collagen I might promote high levels of actomyosin contractility. These mutations might involve alterations that reduce actomyosin contractility or alterations (such as p53 loss) that enable E-cadherin-deficient MECs to survive high levels of actomyosin contractility. Since fibroblasts, the main producers of collagen, infiltrate E-cadherin-deficient neoplastic lesions and massively accumulate in ILC, they likely have conflicting roles depending on the stage of ILC development.

Our results show that relaxation of actomyosin contractility by MYPT1 promotes survival of E-cadherin-deficient MMECs on collagen I and leads to ILC formation. Accordingly, amplification of the MYPT1 homolog MYPT2 or hemizygous deletion of MYH9 are frequently observed in human ILC. The lack of homozygous MYH9 deletions in ILC patients supports the notion E-cadherin-deficient MMECs on collagen I and leads to ILC formation. Nonetheless, we observed that collagen I promotes ILC progression once E-cadherin-deficient MECs have acquired mutations that allow for adhesion and survival on collagen I. These mutations might involve alterations that reduce actomyosin contractility or alterations (such as p53 loss) that enable E-cadherin-deficient MECs to survive high levels of actomyosin contractility. Since fibroblasts, the main producers of collagen, infiltrate E-cadherin-deficient neoplastic lesions and massively accumulate in ILC, they likely have conflicting roles depending on the stage of ILC development.

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In conclusion, this study provides insights into ILC development by uncovering the direct consequences of E-cadherin inactivation in the mouse mammary gland and reveals actomyosin contractility as a critical barrier to ILC development. Moreover, the discovery of actomyosin regulation as a druggable oncogenic pathway might provide therapeutic opportunities to treat E-cadherin mutated cancers.

Methods

Generation of mice. WcreCdhh1F/F;mTmG mice were generated by intercrossing WcreCdhh1F/F mice with mTmG reporter mice. Mice were bred onto an FVB/N background.

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background and genotyped as described\textsuperscript{13,14}. All animal experiments were approved by the Animal Ethics Committee of the Netherlands Cancer Institute and performed in accordance with institutional, national, and European guidelines for Animal Care and Use.

**Intravital imaging.** Mice were anesthetized using isoflurane (1.5% isoflurane/medical air mixture). A small incision was made on top of the ninth and the tenth mammary gland. The gland was mobilized and placed on a coverslip. The mouse is placed in a facemask with a custom designed imaging box. During imaging the mouse received 100 µL/h Nutrilix special 70/240 (Braun) subcutaneous. Imaging was performed on an inverted Leica SP8 multiphoton microscope with a chameleon Vision-S (Coherent Inc., Santa Clare, CA, www.coherent.com), equipped with four HyD detectors: HyD1 (<455 nm), HyD2 (455–490 nm), HyD3 (500–550 nm), and HyD4 (560–650 nm). Different wavelengths between 800 and 960 nm were used for excitation; collagen (second harmonic generation) was excited with a

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**Figure Legends:**

**a** GFP, Laminin, Cytokeratin 8, Merge, Zoom.

**b** Clone: Uncoated, Laminin.

**c** Normalized absorbance

**d** GFP-Collagen.

**e** Vehicle Y27632 Blebbistatin Vehicle Y27632 Blebbistatin

**f** Normalized absorbance

**g** Laminin Collagen I

**h** RhoA-GTP

**i** RhoA expression

**j** Clone: GFP, MYPT1, t-MYPT1, t-MYPT1ΔPP1

**k** Normalized absorbance
**Fig. 6** Collagen I does not support adhesion upon loss of E-cadherin. a IF analysis of GFP (green), Laminin (red), and Keratin 8 (cyan) expression in mammary gland sections from 12-week-old Wcre;Cdh1F/F;MMEC subclones seeded on laminin-coated or collagen I-coated wells in the presence or absence of Y-27632 (10 µM). b, c Representative images (b) and quantification (c) of clonogenic assays with Wcre;Cdh1F/F;MMEC subclones seeded on laminin-coated or uncoated plastic wells. Data are of three independent experiments with three clones per experiment. All data are depicted as mean ± standard deviation. All p values were calculated using an unpaired two-tailed t-test. Source data are provided as a Source Data file.

**Fig. 7** Relaxation of actomyosin contractility drives ILC formation. a Schematic representation of intraductal injections performed in WapCre;Cdh1F/F mice with high-titer lentiviruses produced from vectors encoding MYPT1-wt, t-MYPT1, t-MYPT1ΔPP1, or empty vector (EV). b Quantified tumor burdens of Wcre;Cdh1F/F females 20 weeks after intraductal injection of Lenti-EV (n = 16 glands), Lenti-MYPT1-wt, (n = 12 glands), Lenti-t-MYPT1 (n = 24 glands), or Lenti-t-MYPT1ΔPP1 (n = 14 glands). Data are depicted as mean ± standard deviation. All p values were calculated using an unpaired two-tailed t-test. Source data are provided as a Source Data file.

Intraductal injection of lentiviruses. Intraductal injections were performed as described. Briefly, the mice (2–5 months of age) were anesthetized using ketamine/xylazine (100 and 10 mg/kg, respectively) and hair was removed in the nipple area with a commercial hair removal cream. Eighteen microliters of high-titer lentivirus mixed with 2 µL 0.2% Evans blue dye in PBS was injected in the fourth mammary glands by using a 34G needle. Mice were handled in a biological safety cabinet under a stereoscope. Lentiviral titers ranging from 2 × 10^8 to 2 × 10^9 TU/ML were used. Animals were sacrificed at 20 weeks post-injection. The tumor burden of injected glands was determined as the ratio between the total tumor area and the area of the whole mammary gland, using Fiji (imageJ) software version 1.52n.

Lentiviral vectors and virus production. Generation of the SIN.LV.SF-GFP-T2A-puro (GFP, Fig. 4h–i) and SIN.LV.SF-T2A-puro (Empty Vector, Fig. 4o) was described. MYPT1 was isolated with Age1-Sal1 or Age1-Age1 overhangs and a 3′-UTR pBABE puro vector previously described using Phusion Flash High-Fidelity DNA Polymerase (Thermo Scientific). t-MYPT1 was isolated with Age1–Sal1 or BamH1–Age1 overhangs and a 3′-FLAG tag from the NMuMG cell line RNA using Phusion Flash High-Fidelity DNA Polymerase (Thermo scientific). The cDNA fragments were then inserted into SIN.LV.SF-puro or SIN.LV.SF-T2A-puro to generate SIN.LV.SF-puro vectors.
MYPT1 SIN.LV.SF-t-MYPT1, SIN.LV.SF-MYPT1-T2A-puro, and SIN.LV.SF-t-MYPT1-T2A-puro, and SIN.LV.SF-MYPT1AP1 and SIN.LV.SF-MYPT1AP1-T2A-puro. The pINDUCER20 ref. 59 cassette (with a KRASG12D construct) was isolated with NheI and Sall overhangs using Phusion Flash High-Fidelity DNA Polymerase. The cDNA was inserted into a NheI and Sall digested SIN.LV.SF vector to generate SIN.LV.SF pINDUCER20 KRASG12D. t-MYPT1 was isolated from SIN.LV.SF (MYPT1 with Sall and AgeI restriction enzymes. The AgeI-Sall overhangs were subsequently blunted using Klenow (New England Biolabs). The SIN.LV.SF pINDUCER20 KRASG12D plasmid was digested with EcoRV to remove KRASG12D and inserted with the blunted t-MYPT1 cDNA described above to generate SIN.LV.SF pINDUCER20 MYPT1. All primers are listed in Supplementary Table 1. All vectors were validated by Sanger sequencing. Concentrated lentiviral stocks were produced by transient co-transfection of four plasmids in 293T cells as described. Viral titers were determined using the qPCR lentivirus titration kit from Abm (LV900).

**Cell culture.** Primary MECs were isolated from 10- to 15-week-old females as described and cultured in Dulbecco’s modified Eagle’s medium (DMEM):F12 (10565-018; Gibco) with 10% FBS, 1% penicillin, and streptomycin. The absorbance was normalized to the indicated control samples. The corresponding software. Quanti-stainings were imaged using the Gel count (Oxford Optronix) and its corresponding software version 12.0.0 (Aperio).

**Flow cytomtery.** MEC clones were collected in 1% BSA in 1× PBS and stained with antibodies against: α1 Integrin (1:100; BD Biosciences, 555001), α2 Integrin (1:100; BD Biosciences, 553858), β1 integrin-APC (1:50; Thermo Fischer, 17-0291-82), Cy5-Goat anti Hamster (1:500 Jackson Laboratories), and DAPI (1:20) to exclude dead cells. Samples were analyzed using a BD LSRFortessa Cell Analyzer with BD FACSDiva Software and analyzed using FlowJo software.

**RhoA activation assay.** Fifteen Million cells were plated on regular plastic, collagen-coated or laminin-coated plates and incubated for 3 h at 37°. Cells were washed with PBS and lysed with the lysis buffer included in the RhoA activity assay kit (Cytoselectek, BK036). Lysates were incubated with 30 µg RhoTightin beads for 1 h while rotating. Samples were washed once with 50 µL of 2x sample buffer (Invitrogen, NP0007 and NP0009) was added. Samples were incubated at 95° for 5 min, run on NuPAGE™ 4–12% Bis-Tris Protein Gels (Thermo Fisher, NP0321), and transferred overnight on 0.2 µM nitrocellulose membranes (Bio-rad, 1602112) in transfer buffer (25 mM Tris, 2 M glycine, 20% methanol in demineralized water). Membranes were blocked in 5% nonfat dry milk in TBS-T. Stained membranes were washed three times for 10 min in TBS-T and subsequently developed using ECL (Pierce, 32209). The signal intensities were measured by densitometry in ImageJ and normalized to actin loading controls.

**Laminin and collagen coating.** The laminin coating was generated by letting RAC-11P cells invade the matrigel in 0.5% nonfat dry milk in TBS-T and incubated with the secondary antibody rabbit anti-mouse HRP (1:2000; Dako, P0448). The coated plates were immediately or stored at 4° until use (no longer than 24 h).

**Statistical analyses.** Graphpad Prism v7.03 was used to generate all graphs and perform the statistical analyses. Data are represented as mean and standard deviations. All p values were calculated using an unpaired two tailed t-test.

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

**Data availability** Source data for Figs. 1g, 2c, d, 5d, 5f, j, m, 4b, d, 5d, f, l, k, 6c, e, h, i, k, 7b and Supplementary Figs. 3a-c, 4b and 6b can be found in the source data file. All other data supporting the findings of this study can be obtained from the corresponding authors upon reasonable request.

**Data availability** Source data for Figs. 1g, 2c, d, 5d, 5f, j, m, 4b, d, 5d, f, l, k, 6c, e, h, i, k, 7b and Supplementary Figs. 3a-c, 4b and 6b can be found in the source data file. All other data supporting the findings of this study can be obtained from the corresponding authors upon reasonable request.

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Author contributions
K.S., M.N., A.S., J.V.R., and J.J. designed experiments. K.S., M.N., D.S., V.R, A.P.D., and E.B. performed experiments. M.N. and J.J. supervised the study. K.S., M.N., and J.J. wrote the manuscript.