Outgrowth of single oncogene-expressing cells from suppressive epithelial environments

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Tumorigenesis is a clonal evolution process that is initiated from single cells within otherwise histologically normal tissue. It is unclear how single, sporadic mutant cells that have sustained oncogenic alterations evolve within a tightly regulated tissue environment. Here we investigated the effects of inducing oncogene expression in single cells in organotypic mammary acini as a model to elucidate the processes by which oncogenic alterations initiate clonal progression from organized epithelial environments. Sporadic cells induced to overexpress oncogenes that specifically perturb cell-cycle checkpoints (for example, E7 from human papilloma virus 16, and cyclin D1), deregulate Myc transcription or activate AKT signalling remained quiescent within growth-arrested acini. By contrast, single cells that overexpress ERBB2 initiated a cellular cascade involving cell translocation from the epithelial layer, as well as luminal outgrowth that is characteristic of neoplastic progression in early-stage epithelial tumours. In addition, ERBB2-mediated cell translocation to the lumen was found to depend on extracellular-regulated kinase and matrix metalloproteinase activities, and genetic alterations that perturb local cell–matrix adhesion drove cell translocation. We also provide evidence that luminal cell translocation may drive clonal selection by promoting either the death or the expansion of quiescent oncogene-expressing cells, depending on whether the pre-existing alterations allow anchorage-independent survival and growth. Our data show that the initial outgrowth of single oncogene-expressing cells from organized epithelial structures is a highly regulated process, and we propose that a cell translocation mechanism allows sporadic mutant cells to evade suppressive micro-environments and elicits clonal selection for survival and proliferative expansion outside the native niches of these cells.

The outgrowth of sporadic mutant cells within tightly regulated cellular environments is fundamental to tumour evolution. However, oncogenic alterations are usually not sufficient to predict the behaviours and fates of sporadic cell variants7,8, particularly within complex cellular contexts such as tissues. Limitations in examining single-cell evolution within native tissues have precluded a systematic analysis. Three-dimensional (3D) organotypic cultures recapitulate many of the characteristics of cell dynamics and organization that are found in tissues, while allowing complex manipulations and long-term monitoring at single-cell resolution. MCF10A cells, a non-transformed human mammary epithelial cell line, develop into polarized, growth-arrested acinar structures containing a hollow lumen when cultured on reconstituted basement membrane (Matrigel) (Fig. 1a, b). By modelling the induction of oncogenes in single cells within 3D acini, we explored how sporadic mutant cells evolve within organized epithelial environments.

To induce oncogenes in single cells, growth-arrested (day 16) MCF10A acini that stably expressed the reverse tetracycline transactivator (rtTA) were infected with low-dose lentiviral vectors (pLTI-G) driving the tetracycline (Tet)-inducible bicistronic expression of oncogenes and fluorescent reporters, transducing <0.5% of cells (1 cell per ~10 acini) (Fig. 1b and Supplementary Fig. 2). Overexpression of Myc (also known as c-Myc), a master transcription factor that is deregulated in many cancers, or myrAKT1, which constitutively activates AKT signalling, or perturbation of cell-cycle checkpoints by overexpressing E7 from human papilloma virus 16 (HPV16-E7) or cyclin D1T286X (degradation-resistant cyclin D1) was not sufficient to drive clonal outgrowth. Transduced cells remained quiescent as single cells in the acinar epithelial layer, similar to green fluorescent protein (GFP)-expressing controls (Fig. 1c, d). MCF10A cells that were induced to constitutively express these oncogenes from day 1 of 3D culture developed aberrant hyperproliferative structures (Supplementary Fig. 3), indicating that the lack of clonal expansion from the single-cell context is not due to subthreshold expression. By contrast, overexpression of ERBB2, a receptor tyrosine kinase encoded by a gene that is amplified in 30% of breast tumours9, in sporadic cells within 3D acini effectively drove clonal outgrowth (90 ± 2% of GFP-labelled cell clusters contained multiple nuclei; mean ± s.d.) (Fig. 1c, d and Supplementary Fig. 4). Interestingly, these ERBB2-overexpressing cells developed aberrant hyperproliferative structures (Supplementary Fig. 3), indicating that the lack of clonal expansion from the single-cell context is not due to subthreshold expression. By contrast, overexpression of ERBB2, a receptor tyrosine kinase encoded by a gene that is amplified in 30% of breast tumours, in sporadic cells within 3D acini effectively drove clonal outgrowth (90 ± 2% of GFP-labelled cell clusters contained multiple nuclei; mean ± s.d.) (Fig. 1c, d and Supplementary Fig. 4). Interestingly, these ERBB2-overexpressing...
clones were confined to the lumen (Fig. 1e), resembling the histological feature of early-stage carcinoma-in-situ breast tumours8. Immunostaining for laminin-1, α5, α6, integrin and E-cadherin indicated that gross acinar structures remained intact and that luminal translocation is not associated with epithelial–mesenchymal transition (Fig. 1f). No invasive structures were observed (data not shown). Overexpression of ERBB2 in single cells within 3D acini derived from primary mouse mammary epithelial cells or a highly polarized ovarian cell line, MCAS, also led to luminal localization of the transduced cells (Supplementary Fig. 5). The unique ability of ERBB2 to initiate clonal expansion and the striking pattern of luminal filling suggest that the outgrowth of sporadic mutant cells from organized epithelial structures is tightly regulated.

Long-term (56–85 h) time-lapse confocal microscopy indicated that single GFP-expressing control cells remained quiescent within growth-arrested acini (in 5 of 5 acini). By contrast, single ERBB2-overexpressing cells dissociated from the epithelial layer, showed increased migratory activity and translocated to the lumen (in 6 of 7 acini) (Fig. 2a, Supplementary Movies 1 and 2 and Supplementary Fig. 6). Blocking cell proliferation with aphidicolin did not block translocation (Fig. 2b–d), further indicating that translocation is independent of proliferation. Taken together, these data reveal an initial luminal cell-translocation step in ERBB2-mediated clonal outgrowth.

We next examined the involvement of two major pathways downstream of ERBB2, the mitogen-activated protein kinase (MAPK, or ERK) pathway and the phosphatiidylinositol-3-OH kinase (PI(3)K) pathway, in luminal translocation. MAPK kinase (MEK) inhibition, but not PI(3)K–mammalian target of rapamycin (mTOR) inhibition, greatly reduced ERBB2-mediated translocation (Fig. 2e, f). Moreover, constitutively active MEK (MEK2DD), but not constitutively active AKT (myrAKT1), was sufficient to drive cell translocation to the lumen (Fig. 2g, h). We cannot rule out possible ERK-independent ERBB2 downstream mechanisms, because perturbing ERK activity only partially inhibited or promoted ERBB2-mediated luminal translocation. Recent studies reported that RasV12 and v-Src-transformed Madin–Darby canine kidney (MDCK) cells adjacent to normal neighbours are extruded from monolayer cultures by an ERK-dependent mechanism, although Raf-driven ERK activation is not sufficient to drive extrusion10,11. Another study showed that universal expression of activated Raf induces overall cell motility in MCF10A acini12. Our results suggest that this conserved role of ERK in cell motility is also crucial for ERBB2-mediated single-cell luminal translocation in 3D cultures.

We found that the ERK-regulated transcription factor ETS1 can drive cell translocation to the lumen (Fig. 3a, b). ETS1 transactivates proteins, including matrix metalloproteinases (MMPs)13, which have been implicated in tissue remodelling. Interestingly, MMP inhibition significantly blocked ERBB2-, MEK2DD- and ETS1-induced luminal translocation (Fig. 3c–f).

**Figure 2 | Mechanisms of cell translocation to the lumen.** a. Confocal sections from a time series of acini (nuclei, red) containing single ERBB2-overexpressing cells (green) or GFP-only expressing cells (green) captured starting from approximately 24 h after oncogene induction (indicated as 0 h). b–d. Representative images and quantification of the translocation of ERBB2-overexpressing cells (b, c) and of single ERBB2-overexpressing cells in the lumen (d) of day 16 acini treated with 10 μM aphidicolin (Aph) or dimethylsulphoxide (DMSO) for 8 days. Nuclei were counterstained with DAPI (blue). The nuclei of GFP-expressing clones are outlined (dashed white lines) in some cases to aid visualization. e, f. Representative images (e) and quantification (f) of ERBB2-overexpressing cell translocation in 3D acini treated with a MEK inhibitor (1 μM PD325901 (PD)), a PI(3)K–mTOR inhibitor (20 μM LY294002 (LY)) or DMSO for 8 days. g, h, Representative images (g) and quantification (h) of the luminal translocation of MEK2DD- or myrAKT1-expressing cells from growth-arrested acini. a, b, e, g, Scale bars, 10 μm. c, d, f, h. Data are presented as the mean ± s.d. from four experiments; *, statistically significant difference (two-tailed t-test). a–h. The raw data are shown in Supplementary Table 2.

**Figure 3 | Luminal translocation and clonal outgrowth from a suppressive epithelial environment.** a–c. Representative images (a), quantification of luminal translocation (b) and single cells in the lumen (c) 8 days after induction of ETS1 or MT1-MMP expression (green) in 3D acini. Nuclei were counterstained with DAPI (blue). d–f. Representative images of ERBB2 cells/clones (green) (d) and quantification of translocation (e) and single cells in the epithelial layer (f) of acini treated with the broad-spectrum MMP inhibitors GM6001 or Batimastat, MMP9 inhibitor III (MMP inh. III) or DMSO for 8 days. The nuclei of GFP-expressing clones are outlined (dashed white lines) in some cases to aid visualization. g, h. Representative images (g) and quantification (h) of translocation of cells in which talin 1 expression was knocked down (talin-1 KD) (red) in 3D acini. Immunoblotting (h, right) indicates efficient knockdown of talin-1 expression. Similar results were obtained with a different talin-1 knockdown construct (data not shown). NS, non-silencing construct. a, d, g. Scale bars, 10 μm. b, c, f, h. Data are presented as the mean ± s.d. from three to four experiments; *, statistically significant difference (two-tailed t-test). a–h. The raw data are shown in Supplementary Table 3.
translocation (Fig. 3d, e and Supplementary Fig. 7). Although the identity of the MMPs involved is unclear, these data indicate that MMP activity is important for cell translocation. We overexpressed MT1-MMP (also known as MMP14) to examine whether MMP activity can promote translocation. MT1-MMP was chosen because its membrane localization and broad substrate specificity make it an attractive tool for modulating local MMP activity. Single-cell MT1-MMP overexpression in MCF10A acini was sufficient to drive cell translocation to the lumen. Notably, neither ETS1-induced translocation nor MT1-MMP-induced translocation drove clonal expansion (Fig. 3a–c), and both were independent of ERK signalling (Supplementary Fig. 8). Taken together, these results identify specific proliferation-independent pathways that promote cell translocation to the lumen (Fig. 3a, b).

We proposed that perturbation of local cell–matrix adhesion by MMPs could trigger cell translocation. Consistent with this idea, MCF10A cells overexpressing ERBB2, but not Myc, myrAKT1 or GFP, showed impaired adhesion to Matrigel-coated plates (Supplementary Fig. 9). In addition, we observed compromised basement membrane adjacent to single ERBB2-overexpressing cells, but not adjacent to Myc-, myrAKT1- or GFP-overexpressing cells, in 3D acini (Supplementary Fig. 9). Moreover, weakening the cell–matrix adhesion strength by knocking down expression of the gene encoding talin 1 (ref. 14), a protein that links integrins to actin filaments and localizes to the basal cell membrane in 3D acini (Supplementary Fig. 10), was sufficient to induce translocation (Fig. 3g, h).

Intriguingly, the ERBB2-overexpressing cells that stayed in the epithelial layer as a result of MMP inhibition were unable to proliferate (Fig. 3f and Supplementary Fig. 11). MMP inhibition did not affect the proliferation of ERBB2-overexpressing cells in monolayer cultures, and the treatment of acini with an MMP inhibitor 4 days after single-cell ERBB2 induction, when most induced cells had already translocated, did not affect luminal outgrowth (Supplementary Fig. 12), suggesting that MMP activities are required specifically for the initial translocation step but not for proliferation.

Cell displacement has been proposed as a mechanism for removing aberrant cells from epithelia11,17,18. Our data predict that cell displacement by translocation may also facilitate the outgrowth of sporadic mutant cells. Using MT1-MMP as a tool to drive cell translocation, and myrAKT1- and Myc-overexpressing cells as models, we examined the outcome of forced translocation of quiescent oncogene-expressing cells. Single cells within MCF10A acini that stably carried Tet-inducible MT1-MMP-IRES-GFP (pLT-MT1-MMP-iGSP) or IRES-GFP (pLT-iGSP) vectors were infected with another lentiviral vector encoding Tet-inducible myrAKT1-IRES-mCherry or Myc-IRES-mCherry as well as constitutive rTA expression (pLT-myrAKT1-iCSA or pLT-Myc-iCSA). Only these transduced single cells contain all of the Tet-inducible components required to drive doxycycline-dependent expression of myrAKT1 (or Myc), MT1-MMP and the two fluorescent reporters (Supplementary Fig. 13). This combinatorial inducible approach overcomes the size limitations on efficient virus packaging and allows flexible, multiplex genetic manipulations in single cells.

Forced translocation of cells co-expressing myrAKT1-mCherry and MT1-MMP-GFP, but not cells co-expressing Myc-mCherry and MT1-MMP-GFP (or mCherry and MT1-MMP-GFP), led to luminal expansion (Fig. 4a, b). Cells co-expressing myrAKT1-mCherry and MT1-MMP-GFP that failed to translocate remained as single cells in the epithelial layer (99 ± 2% of GFP-labelled cells; mean ± s.d., indicating that translocation, but not simply MT1-MMP and myrAKT1 co-expression, facilitates clonal outgrowth. Indeed, MT1-MMP did not increase the colony formation of myrAKT1-expressing MCF10A cells in soft agar (Supplementary Fig. 14).

Both translocated Myc-overexpressing cells and myrAKT1-overexpressing cells re-entered the cell cycle (Fig. 4c), but only Myc-overexpressing cells showed increased apoptosis (Fig. 4d, e). These results suggest that the anti-apoptotic activity of myrAKT1 contributes to support clonal expansion in the matrix-deprived lumen. Consistent with these observations, 42 ± 8% and 29 ± 12% (mean ± s.d.) of translocated ETS1-overexpressing and MT1-MMP-overexpressing single cells, respectively, also underwent apoptosis (Fig. 4e). Although we could not directly trace these cells, the apoptosis of single translocated ETS1-, MT1-MMP- or Myc-overexpressing cells suggests that the clonal lineage would probably be eliminated.

This dichotomous fate of Myc-overexpressing cells and myrAKT1-overexpressing cells suggests that translocation is not sufficient for outgrowth but instead might unleash cells from their suppressive epithelial environment. We tested whether perturbing the epithelial organization of 3D acini allows the expansion of quiescent mutant cells. Knockdown of CTNND1 (which encodes p120-catenin), to destabilize the epithelial cell–cell junctions18 in preformed acini, greatly reduced staining for β-catenin and E-cadherin at cell junctions, without disrupting gross acinar structures (Supplementary Fig. 15). In contrast to the distinct outcomes of forced translocation, both Myc-overexpressing cells and myrAKT1-overexpressing cells, but not GFP-expressing cells, underwent clonal expansion on p120-catenin

**Figure 4 | Cell translocation elicits clonal selection or outgrowth of quiescent mutant cells.** a–d. Single cells in day 16 MCF10A pLT-MT1-MMP-iGSP- (top) or pLT-iGSP (IRES-GFP)-infected acini (bottom) were infected with pLT-Myc-iCSA, pLT-myrAKT1-iCSA or pLT-iCSA (IRES-mCherry) to inducibly drive oncogene and reporter co-expression. Nuclei were counterstained with DAPI (blue). Representative images (a) and quantification (b) of Myc and mCherry, myrAKT1 and mCherry, or mCherry cells/clones co-expressing either MT1-MMP and GFP or GFP only (yellow) 12 days after doxycycline induction. Nuclei of clones co-expressing MT1-MMP and myrAKT1 are outlined (white dashed line). Quantification of proliferation (c) and apoptosis (d) in translocated cells/clones. e. Acini with cells overexpressing ETS1 or MT1-MMP or with cells co-expressing Myc and MT1-MMP were immunostained with antibody specific for cleaved caspase 3 8 days after induction. f, g. Single cells within preformed, day 16 MCF10A pTRIPZ-p120KD- or pTRIPZ-NS (non-silencing)-expressing acini were infected with pLT-Myc-iG, pLT-myrAKT1-iG, pLT-ERBB2-iG or pLT-iG. Acini were induced with doxycycline to drive expression of a p120-catenin knockdown (p120 KD; red) or non-silencing (NS; red) short hairpin RNA in all cells with co-expression of Myc, myrAKT1, ERBB2 or GFP (all in green) in single cells. Acini were induced with doxycycline for 48 h to express the p120-catenin KD or non-silencing short hairpin RNA before infection with pLT-ERBB2-iG. Representative images (f) and quantification (g) of expanded clones in the epithelial layer 8 days after induction. a, e, f. Scale bars, 10 μm. b, d, g. Data are presented as the mean ± s.d. from four experiments; *, statistically significant difference (two-tailed t-test). a–g. The raw data are shown in Supplementary Table 4.
downregulation (Fig. 4f, g). These data suggest that the epithelial organization mediated by p120-catenin and cadherin junctions is crucial for suppressing oncogene-induced proliferation in mature acini. Notably, single ESRB2-overexpressing cells in preformed acini that were subjected to p120-catenin knockdown also proliferated but did not translocate (Fig. 4f, g), suggesting that intact epithelial organization has a role in supporting cell translocation to the lumen.

These findings highlight the suppressive influence of a mature epithelial environment on sporadic mutant cells and raise the question of whether the expression of oncogenes in neighbouring cells would abrogate this proliferative suppression. Interestingly, induction of Myc or myrAKT1 in all cells of growth-arrested acini did not drive cell proliferation or disrupt acinar structure (Supplementary Fig. 16). Taken together, these results further demonstrate the strong suppressive control of organized epithelium, because an oncogene such as Myc or myrAKT1 is not sufficient to abrogate this suppressive effect.

We used 3D organotypic cultures to model the genetic and tissue architectural context in which sporadic oncogene-expressing cells arise in the early stages of human breast tumorigenesis, and we demonstrated that the initial outgrowth of these sporadic mutant cells within organized epithelial environments is highly regulated. We showed that although perturbation of a suppressive epithelial environment allows a general expansion of quiescent mutant cells, a different process, involving cell translocation to the lumen, allows mutant cells to evade the suppressive epithelial environment and drives selection for survival and expansion in the matrix-deprived lumen (a model is shown in Supplementary Fig. 1). Our data also highlight the suppressive influence of organized epithelial structures on pre-neoplastic cell expansion.

The displacement of cell variants from epithelia and stem cell compartments has been widely observed in diverse organ systems. Previous studies have shown that the extrusion of apoptotic cells from epithelial monolayers involves a force-dependent expulsion process from neighbouring cells. We show that cell translocation to the lumen in 3D cultures is induced by ERK and MMP activities that are intrinsic to the translocating cells and involves local perturbations of cell–matrix adhesion.

The migration of cells from specialized niches and micro-environments underlies cell differentiation and tissue morphogenesis during development and regeneration. Our data suggest that a similar spatial cell translocation within tissue compartments may also have a role in tumorigenesis by eliciting clonal selection. Oncogenic alterations have been observed in cells in otherwise histologically normal tissue of tissue compartments may also have a role in tumorigenesis by eliciting clonal selection. Oncogenic alterations have been observed in cells in otherwise histologically normal tissue of healthy individuals, and the disruption of tissue organization has been associated with tumour progression. Our findings raise the possibility that mechanisms such as cell translocation or compromised tissue architecture on sporadic mutant cells would abrogate this proliferative suppression. Interestingly, induction of Myc or myrAKT1 in all cells of growth-arrested acini did not drive cell proliferation or disrupt acinar structure (Supplementary Fig. 16). This observation is consistent with a previous finding that tamoxifen-reversal of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J. Cell Biol. 137, 231–245 (1997).

METHODS SUMMARY

3D Matrigel culture and virus infection. MCF10A cells were set up in 3D cultures on basement membrane in 8-well chamber slides (BD Biosciences) or coverglass-bottom 8-chamber slides (MatTek) as previously described, with 4,500–5,000 cells in assay medium (DMEM/F12 supplemented with 2% donor horse serum, 5 mg ml⁻¹ epidermal growth factor (EGF), 10 µg ml⁻¹ insulin, 1 ng ml⁻¹ cholera toxin, 100 µg ml⁻¹ hydrocortisone, 50 µU ml⁻¹ penicillin, 50 µg ml⁻¹ streptomycin and 2% Matrigel). The medium was replaced at 4-day intervals. On day 16, 3D cultures were infected with the indicated lentiviruses diluted in assay medium without EGF or Matrigel and incubated for 6–8 h at 37 °C. Virus dosages were adjusted to infect less than 1 cell per 10 acini, to achieve sporadic single-cell infection. The virus was removed, and the culture wells were rinsed with 500 µl PBS, which was replaced with normal 3D assay medium without Matrigel. Doxycycline (1 µg ml⁻¹) was added on the following day, together with drug treatment or vehicle control as indicated. Drugs and vehicles were replenished at 2-day intervals, and the complete medium was changed at 4-day intervals.

Full Methods and any associated references are available in the online version of the paper at the online site of nature.com/nature.

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METHODS

Cell culture. MCF10A cells were cultured as described previously25. MCAS cells were cultured in 1:1 Medium 199:MCDB 105 medium with 2 mM l-glutamine, 10% heat-inactivated FCS and 100 U ml⁻¹ penicillin and streptomycin. Primary mouse mammary epithelial cells (MECs) were cultured in DMEM/F12 with 5% FBS, 5 µg ml⁻¹ insulin, 1 mg ml⁻¹ hydrocortisone, 3 ng ml⁻¹ EGF and 100 U ml⁻¹ penicillin and streptomycin.

Chemicals. Doxycycline was used at 1 µg ml⁻¹. The following doses of inhibitors were used: 20 µM LY294002, 1 µg ml⁻¹ PDI325901, 10 µM GM6001, 5 µM Batimatastat, 5 µg ml⁻¹ MMP2/MMP9 inhibitor III and 10 µM aphidicolin.

Lentiviral vectors. The vectors pBABE-hygro-rTA and pBABE-puro-rTA were constructed by subcloning the coding sequence of rTA from PpTet-On-Advanced (Clontech) into the pBABE-hygro or pBABE-puro retroviral vectors, respectively. The vector pBABE-neo-MTI-MMP was constructed by amplifying the coding sequence of MT1-MMP (Invitrogen) using PCR and subcloning it into pBABE-neo. The vector pBABE-puro-H2B-mCherry was constructed by amplifying the coding sequence of amino acids 1–125 of the human H2B gene using PCR, fusing it to the amino terminus of mCherry (Clontech) and subcloning it into pBABE-puro.

Lentiviral vectors. The pLITg-lentiviral inducible vector was constructed by replacing the CAG promoter of the pCSG-SP-PW lentiviral vector (Addgene, plasmid 12335) with the tetracycline response element (TRE) from pTet-Tight (Clontech) and inserting a downstream IRES-GFP cassette from pRES2-GFP (Clontech). The coding sequences from the following sources were either directly subcloned or PCR-amplified and then subcloned between the TRE and IRES-GFP cassette of pLITg-iG. ERBB2 (pBABE-puro-ERBB2), myrAKT1 (pBABE-puro-myrAKT1), MEK2DD (pBABE-puro-MEK2DD) and HPV16-E7 (plcCNX-E7) were obtained as previously described26,27. CyclinD128 (pDNA-CyclinD1 HA HA-T286A, plasmid 11182) and Myc (pcDNA3-Myc, plasmid 16011) were obtained as previously described25,26. CyclinD1 was confirmed by immunoblotting after induction for 2 days in 3D assay medium with 1 µg ml⁻¹ doxycycline on monolayer culture cells. The following antibody dilutions were used: α-integrin (1:1,000, Millipore), laminin–γ2 (1:100, BD Biosciences), GM130 (1:100, Cell Signaling Technology), E-cadherin (1:100, Cell Signaling Technology), β-catenin (1:1,000, Cell Signaling Technology), GFP (1:100, Invitrogen), activated caspase 3 (1:100, Cell Signaling Technology), Ki67 (1:100, Invitrogen), ERBB2 (1:100, Cell Signaling Technology), ZO1 (1:100, Invitrogen) and talin 1 (1:1,000, Abcam). Alexa-Fluor-conjugated secondary antibodies (Invitrogen) were used at 1:200–1:100 for 2 h at room temperature. Nuclei were counterstained with DAPI. Images were acquired using Nikon CI or Nikon A1 confocal microscopes.

Immunoblotting. Inducible overexpression or knockdown of the target genes was confirmed by immunoblotting after induction for 2 days in 3D assay medium with 1 µg ml⁻¹ doxycycline on monolayer culture cells. The following antibody dilutions were used: HPV16-E7 (1:100, Zymed), ERBB2 (1:1,000, Cell Signaling Technology), pan-AKT (1:500, Cell Signaling Technology), cyclin D1 (1:500, Cell Signaling Technology), Myc (1:1,000, Cell Signaling Technology), p120-catenin (1:1,000, BD Biosciences) and talin 1 (1:1,000, Cell Signaling Technology). β-Tubulin was used as a loading control.

Soft-agar colony formation assay. Cells (50,000) were seeded in 0.4% soft agar in normal MCF10A growth medium on a layer of 0.5% soft agar in normal growth medium. Cultures were fed every 6 days with 1 ml of 0.4% soft agar in normal MCF10A growth medium on a layer of 0.5% soft agar in normal growth medium. Cultures were fed every 6 days with 1 ml of 0.4% soft agar in normal growth medium.

Growth curve. ERBB2-overexpressing MCF10A cells or empty vector control cells were seeded at 100,000 cells per well in 6-well plates for 48 h in 3D assay medium with 25 µM GM6001 or dimethylsulphoxide (DMSO). The cell doubling rate was averaged from three individual experiments.

Adhesion assay. The expression of ERBB2, Myc, myrAKT1 or GFP was induced in MCF10A monolayer cultures for 72 h, and the cells were then trypsinized, plated at 30,000 cells per well in 24-well Matrigel-coated plates and incubated at 37 °C for 1 h. The plates were washed three times with PBS, and the cells were fixed with paraformaldehyde. Attached cells were counterstained with DAPI. The average number of attached cells from four arbitrary 20× fields in the centre of the wells was calculated from three individual experiments.

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