High cell-surface density of HER2 deforms cell membranes

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Breast cancers (BC) with HER2 overexpression (referred to as HER2 positive) progress more aggressively than those with normal expression. Targeted therapies against HER2 can successfully delay the progression of HER2-positive BC, but details of how this overexpression drives the disease are not fully understood. Using single-molecule biophysical approaches, we discovered a new effect of HER2 overexpression on disease-relevant cell biological changes in these BC. We found HER2 overexpression causes deformation of the cell membranes, and this in turn disrupts epithelial features by perturbing cell-substrate and cell-cell contacts. This membrane deformation does not require receptor signalling activities, but results from the high levels of HER2 on the cell surface. Our finding suggests that early-stage morphological alterations of HER2-positive BC cells during cancer progression can occur in a physical and signalling-independent manner.

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**HER2** is a member of the ErbB/HER receptor tyrosine kinase family\(^1\)-\(^3\). Gene amplification and overexpression of this protein in breast cancers (BCs) predict poor disease outcome due to elevated metastatic potentials\(^4\)-\(^7\). Studies of the role of HER2 in cancer progression have focused primarily on the signalling activities of HER2. HER2 BC cells with a 3 \(\pm\) immunohistochemistry (IHC) score exhibit high basal levels of receptor kinase activity and phosphorylation, and these induce constitutive activation of the mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways\(^8\). These activities can result in increased cell proliferation\(^9\),\(^10\) and invasiveness\(^1\). Moreover, HER2 3 \(\pm\) BC cells exhibit upregulated epithelial-mesenchymal transition (EMT) inducing transcription factors, such as TWIST and SNAIL, through which cancer cells lose their epithelial characteristics\(^12\).

These characteristics of HER2 overexpression were acquired from various ensemble experiments, where receptor activities are typically averaged over a very large number of cells. In our previous studies of epidermal growth factor receptor (EGFR) dimerization dynamics at a single-molecule level, we showed that receptor retention by ligand binding is spatially asymmetric on cells overexpressing EGFR (ref. 13). Thus we considered the possibility that individual HER2s may also behave non-uniformly within a single BC cell, and that this heterogeneity might bear a biological significance. Therefore, we employed quantum dot (QD)-based single-receptor tracking and analysis methods in live cells to investigate whether a spatial control is present that may influence the activation of overexpressed HER2s in BC cells. We found that HER2s were distributed in clusters with elongated shapes on cells overexpressing the receptor, while the distribution was more uniform when the expression level was normal. Interestingly, this clustered distribution was independent of HER2 signalling. We found that these patterns resulted from deformed membrane morphologies, which appeared as irregularly shaped ‘finger-like’ structures (FLS) in electron micrograph images of HER2 3 \(\pm\) BC cells grown in vitro as well as in tissue samples from BC patients. Surprisingly, these finger-like membrane structures were also observed in cells overexpressing signalling-incompetent HER2 mutants, suggesting membrane deformation is induced by the high cell-surface density of HER2 rather than by the receptor’s signalling activities. We found that this membrane deformation can reduce the area available for cell contacts with substrates or neighbouring cells. These observations suggest that a non-canonical effect of HER2 overexpression exists that contributes to the disruption of epithelial characteristics exhibited in HER2 3 \(\pm\) BC cells\(^14\), which is implicated in early-stage cancer progression\(^15\),\(^16\).

### Results

**Elongated and clustered HER2 distribution in high expressers.**

We examined the spatial distribution of individual HER2s on live cells that express different levels of HER2. The cells were grouped according to the IHC scoring system, where 0 represents normal level expression, 1 \(\pm\) and 2 \(\pm\) are mid, and 3 \(\pm\) is high expression. A large number of location points of individual HER2s were obtained by tracking single receptors (for \(\sim 100\) s at a 10.72 Hz acquisition rate) labelled with anti-HER2 Fab:QD (\(\sim H2Fab:QD\)) conjugates using total internal reflection fluorescence microscopy (TIRFM)\(^13\). We first compared the receptor distribution patterns within the same cell type where HER2 expression levels were artificially altered. Pairs of low and high HER2 expressers were created for two BC cell lines (Fig. 1a). MCF-7 is a low expresser and MCF-7-HER2 is a stable line engineered to overexpress HER2 (3 \(\pm\)). SK-BR-3 naturally overexpresses HER2 (3 \(\pm\)) and its HER2 level was reduced (to \(\sim 1\) \(\pm\)) by partial small interfering RNA (siRNA) knockdown (siH2-SK-BR-3). To compare the HER2 distribution patterns between cells with widely differing receptor expression levels, the number of receptor locations per image was kept constant (variation \(< 3\%\)) by adjusting the concentration of \(\sim H2Fab:QD\) conjugates. As a result, all images in Fig. 1 contain \(\sim 60,000\) receptor location points in each \(8 \mu m \times 8 \mu m\) field. Surprisingly, a large fraction of HER2s in the two high expressers appeared ‘clustered’ in elongated shapes, whereas the HER2s in their low-expression counterparts were more uniformly distributed and only a few circular ‘clusters’ were found (Fig. 1a). (It should be noted that the term ‘clusters’ describes regions on the membrane with high optical densities of HER2, but this does not necessarily correspond with actual receptor clustering.)

We performed similar experiments on other BC cell lines with varying HER2 levels (listed in Fig. 1b) to explore whether this visual ‘clustering’ of receptor locations is a general phenomenon in high HER2 expressers. Indeed, the elongated cluster patterns of HER2 were frequently observed in other high expressers (EEM-192A, BT-474 and KPL-4), where less frequent in mid expressers (MDA-MB-175 VII (1 \(\pm\)) and MDA-MB-453 (2 \(\pm\))) and rarely observed in low expressers (BT-549, MDA-MB-436, MDA-MB-231X1.1 and MCF10DCIS.com). (HER2 expression levels are compared by WB in Supplementary Fig. 1a). Examples of the HER2 distribution patterns in these cells are shown in Fig. 1b and Supplementary Fig. 1b. We found the receptor level-dependent location patterns are not unique to HER2 as overexpression of EGFR in MCF-7 also generated elongated cluster patterns (Supplementary Fig. 1c).

The elongated HER2 distribution patterns were quantified using a ‘cluster analysis’ algorithm that we created. This analysis identifies clustered receptor locations by hierarchically grouping location points that maintain a four-point nearest neighbour connectivity, and enables characterization of the cluster shapes (see Methods section and Supplementary Fig. 2a, for details; very little clustering was found from location points simulated from random walker trajectories). As shown in Fig. 1c, the number of clusters per unit area (8 \(\mu m \times 8 \mu m\)) was significantly greater in high expressers than in mid and low expressers. Since the clusters look more elongated in high expressers (Fig. 1a,b), we characterized the extent of elongation of each cluster by calculating its isoperimetric quotient (IQ), which is the ratio of the area of a given cluster to that of a circle with the same perimeter length. The IQ value for a circle is 1 and decreases as the shape becomes more elongated. As expected, the average IQ was generally smaller for high HER2 expressers than for lower expressers (Supplementary Fig. 2b). Figure 1c shows that high expressers contain significantly more elongated clusters with IQ values smaller than 0.25 compared with those for the low expressers. (This threshold value was determined from the intersection point of the distribution curves of IQ values in high and low HER2 expressers, Supplementary Fig. 2c).

Notably, single-molecule tracking revealed that most HER2s within the elongated clusters were not entirely stationary but could diffuse around within and between the clusters (Supplementary Fig. 2d). This observation suggests that the apparent ‘cluster’ of HER2 locations does not indicate an actual cluster (aggregation) of a large number of HER2s; but is instead a result of a confined geometry of the membrane within which these receptors could diffuse.

**Membrane deformation is observed in high HER2 expressers.**

We mapped out the three-dimensional (3D) distributions of HER2 to better characterize their location patterns by confocal microscopy. The 3D distributions of QD-labelled HER2s on live
MCF-7 (low expresser) and MCF-7-HER2 (high expresser) cells were created along the optical (z) axis for the apical and basal membranes. Side views of these z-stack images (Fig. 2a) show irregular and thick HER2 distributions in MCF-7-HER2s (green) in both apical (top panel) and basal (lower panel) membranes. This is in contrast to the overall thin and more planar distributions observed in MCF-7s (yellow) on both surfaces. Interestingly, the 3D shapes of the HER2 locations in MCF-7-HER2s appear to form narrow and elongated structures. One possible explanation for the thick HER2 distribution along the z axis in MCF-7-HER2s is receptor internalization. However, the majority of zH2Fab:QD conjugates attached to HER2s on MCF-7-HER2s were removed by a 10 min wash with 2 M urea/50 mM glycine (pH 2.4)17 (Fig. 2b). This indicates that most HER2s were membrane bound and exposed to the cell exterior, rather than located inside the cell. In other words, the narrow and elongated 3D receptor location patterns (Fig. 2a) reflect deformed cell membrane morphologies.

If a cell membrane forms an elongated 3D structure, its two-dimensional (2D) optical projection may consist of multiple membrane layers (see the cartoon in Supplementary Fig. 2e). Then, objects associated with the membrane would appear at higher densities in the layered regions, and this could explain the clustered patterns observed in HER2 overexpressers. To test this hypothesis we stained low and high HER2 expressers with a lipophilic dye (1,10-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) that becomes non-specifically incorporated into the membrane. We then compared the fluorescence intensity patterns of this dye in the cell membranes of low and high expressers (Fig. 2c). Numerous higher intensity regions (orange) are observed in the basal membranes of MCF-7-HER2 cells (right), while only a few high intensity regions are found in MCF-7s (left). IQ values of the high intensity membrane regions were smaller (ie, more elongated) in MCF-7-HER2 than in MCF-7 cells (Supplementary Fig. 2f). This was consistent with the patterns of clustered HER2 locations in these cells (Fig. 1a,c), which we also observed from the fluorescence intensity pattern of HER2-GFP when it is overexpressed in MCF-7 cells (Supplementary Fig. 2g). The similar patterns of HER2 and the membrane dye suggest the HER2 clusters likely reflect regions of membrane folding.

**Figure 1 | Contrasting HER2 distributions in high versus low expressers.** (a) A HER2 distribution was constructed from the sum of individual receptor location points (black dots) acquired from tracking single HER2s in the basal cell membrane, for 100 s with an acquisition rate of ∼11 Hz. Each distribution contains ∼60,000 location points in the 8 μm × 8 μm field. It was assumed that the membrane as a whole was stationary during this time window. In high HER2 expressers, MCF-7-HER2 and SK-BR-3 (3+, red circle), HER2 locations appeared ‘clustered’ in elongated shapes while they are randomly distributed in the low expressers, MCF-7 and siH2-SK-BR-3 (light grey circle). (b) Elongated HER2 clusters are also observed in high expressers (red circle, KPL-4), much less in the mid (light blue circle, MDA-MB-175-VII) and almost invisible in low expressers (light grey circle, MDA-MB-436). All fields are 8 μm by 8 μm. The list of BC cell-lines with different HER2 levels that are used in c and d). (c) The numbers of clusters (mean ± s.e.m.) identified in these fields. (d) The numbers of elongated clusters (mean ± s.e.m.) with the IQ values <0.25. ***P value<10−6 by two-sided, unpaired Welch’s t-test.
**Figure 2 | Membrane deformation by high HER2 expression.** (a) The 3D distributions of QD-labelled HER2s in the apical (top, side view) and basal membranes (bottom, side view) of MCF-7 (yellow) and MCF-7-HER2 (green) created from z-stack snap-shot fluorescence images using confocal microscopy (400 ms bin time). In MCF-7-HER2, HER2s are broadly distributed along the z axis in elongated structures on both apical and basal sides, and the 2D projection of these morphologies appears as elongated clusters in the basal membrane as shown in b (before urea/acid wash). Scale bar, 3 μm. (b) The 2D projection of z-stack images of HER2 locations (green) from the basal membrane to ~3.3 μm inside of MCF-7-HER2 cells. Most of the αH2Fab:QD conjugates initially bound to HER2s (left) were removed by a 10 min urea/acid wash (right). Scale bar, 3 μm. (c) High fluorescence intensity regions of the membranes stained with a lipophilic dye (orange) in live MCF-7 and MCF-7-HER2 cells. Inserts: raw fluorescence images inside the boxes. Scale bar, 4 μm. (d) TEM images of different cell membrane morphologies of MCF-7 and MCF-7-HER2. FLS of cell membranes are observed throughout the entire surface of MCF-7-HER2 cells. Zoomed-in images of MCF-7 inside white boxes are shown in Supplementary Fig. 3a. Scale bar, 2 μm. Panel e is the zoomed-in (~8 times larger) image of the region in the white box in d. Scale bar, 0.2 μm. (f) Bud-like membrane protrusions (violet arrows) are observed in MCF-7-HER2. Scale bar, 0.2 μm. (g) The width (the distance between two orange arrows) of the elongated clusters of HER2 locations (white dots) is ~0.18 μm (n = 200), which is similar to that of the FLS in e. The precision of determining the centre position of a single QD is <4 nm, similar to the resolution of the EM. Scale bar, 0.2 μm.

We used transmission electron microscopy (TEM) to directly visualize the membrane morphologies of intact MCF-7 and MCF-7-HER2 cells. In MCF-7 cells, the membrane overall is smooth with a few long, irregularly shaped protruded structures (black arrows) on both the apical and basal sides (Fig. 2d, left; zoomed-in images of the areas inside the white rectangles are shown in Supplementary Fig. 3a). In contrast, a larger number of elongated membrane structures, which show association with HER2s (Supplementary Fig. 3b), are observed in MCF-7-HER2 (right), and SK-BR-3 cells (Supplementary Fig. 3c) throughout the entire cell surface. We refer to these protrusions as ‘FLS.’ The enlarged image (Fig. 2e) of the area inside the white rectangle in Fig. 2d (right) clearly displays FLS (green arrows), which are very similar to the protrusive cell membrane structures of SK-BR-3 observed by Hommelgaard et al.18. The FLS were often not significantly associated with actin filaments (Fig. 2e). Besides actin, other actin binding proteins such as villin and ezrin, and phosphatidylinositol (4,5) bis-phosphate (PIP2) that can be associated with these proteins showed little (~20%) overlap with FLS (immuno-fluorescence (IF) images in Supplementary Fig. 3d–g), suggesting the membrane deformation in these cells was not caused by actin-mediated protrusion in a manner analogous to the brush-border formation19. The FLS were also unrelated to caveolin-1 (cav1) mediated membrane tubulation (invagination) that only occurs after cav1 transfection in SK-BR-3 (ref. 20). Interestingly, small bud-like membrane protrusions were frequently observed, which may represent an early-stage in the process that creates FLS (Fig. 2f and Supplementary Fig. 3b). Lateral widths (mean ± s.d.; indicated by orange arrows in Fig. 2e,g) of FLS (0.15 ± 0.05 μm) in TEM images of MCF-7-HER2 cells were very similar to those determined for the elongated HER2 clusters (0.18 ± 0.05 μm) obtained from the single-receptor tracking measurements (Fig. 2g).

Membrane deformation is independent of HER2 signalling. HER2s can become constitutively active when overexpressed. We thus questioned whether the membrane deformation was a consequence of constitutive receptor signalling. To address this possibility, we constructed three different signalling-incompetent
mutants of HER2. The three mutations were (i) a point mutation in the kinase domain that disrupts ATP binding (knHER2), (ii) phenylalanine substitutions for seven individual tyrosine auto-phosphorylation sites (7YFHER2), and (iii) deletion of the entire intracellular domain of the receptor (ΔicdHER2). We generated MCF-7 transfectants that stably expressed knHER2 and ΔicdHER2, along with wtHER2 at high levels (3⁺). We also used all three mutants and wtHER2 for transient expressions in MCF-7 cells. Surprisingly, all MCF-7 cells overexpressing these mutants and wtHER2 exhibited elongated and clustered location patterns of HER2s, while MCF-7-vctrl (transfectants of the control vector) showed random patterns (representative HER2 distributions in the stable and transient transfectants are respectively shown in Fig. 3a and Supplementary Fig. 4a). These results suggest the membrane deformation in high expressers is independent of HER2 signalling. We also examined whether blocking the HER2 kinase activity affects existing FLS by treating the high-expresser MCF-7-wtHER2 with lapatinib, a kinase inhibitor of HER2, for 30 min (93% decrease in HER2 phosphorylation relative to control as assessed by WB, Supplementary Fig. 4b). Despite the reduced HER2 kinase activity, elongated and clustered receptor location patterns in MCF-7-wtHER2 were unaffected overall.

Figure 3 | Membrane deformation by HER2 overexpression is independent of HER2 signaling. (a) Elongated HER2 clusters are observed in high expressers of signalling-incompetent HER2 mutants, MCF-7-knHER2 and MCF-7-ΔicdHER2, similar to those in wtHER2 high expressers (orange diamond). Clustered HER2 location patterns shown in MCF-7-wtHER2 (orange diamond) were also largely insensitive to 30’ kinase inhibitor (lapatinib) treatment (grey diamond). The occurrence of the elongated HER2 clusters in MCF-7-vctrl (grey diamond) is significantly less. (b) Numbers of elongated clusters quantified by cluster analysis in MCF-7 and SK-BR-3 cell variants, indicate that regardless of HER2 signalling activities, 3⁺ HER2 expression results in significantly greater FLS than low expression (MCF-7-vctrl and siH2-SK-BR-3 cells). Light grey/green/purple circles: low/high/high HER2 levels and low/high/low HER2 signalling activities. (c) Effects of 1 day treatments of MCF-7-HER2 cells with trastuzumab fab (Tfab), trastuzumab (Tmab), pertuzumab fab (Pfab) and pertuzumab (Pmab) on elongated cluster patterns of HER2. Tfab and Pfab treatments noticeably down-modulated clustered HER2 patterns in MCF-7-HER2. (d) Comparison of the numbers of elongated clusters in MCF-7-HER2 and SK-BR-3 cells untreated/treated with Tfab, Tmab, Pfab and Pmab. Green/magenta/blue circles: untreated/Tfab and Tmab treatments/Pfab and Pmab treatments. ***P value <10⁻⁶ and **P value <10⁻⁴ by two-sided, unpaired Welch’s t-test. All values are mean ± s.e.m.
The membrane deformation in SK-BR-3 was also largely insensitive to reduced HER2 signalling, as elongated cluster patterns of HER2 locations (Supplementary Fig. 4c) as well as FLS in TEM images (Supplementary Fig. 3c), persisted after lapatinib treatment.

Using cluster analysis, we compared the FLS densities in cells expressing 3+ levels of the various signalling-incompetent mutants with those of wtHER2 by calculating the average number of elongated clusters per unit area (8 μm by 8 μm). FLS densities in all 3+ expressers are comparable, and significantly higher than those in their low-expresser counterparts (MCF-7-vctrl and siH2-SK-BR-3). The results in Fig. 3b (for stable transfectants) and Supplementary Fig. 4a (transient transfectants) clearly indicate that FLS formation and maintenance depend on the presence of high HER2 expression in the cell membrane rather than receptor signalling activities.

**HER2 antibodies can affect membrane deformation.** Although the membrane deformation is not sensitive to inhibition of HER2 kinase activity, we questioned whether the binding of therapeutic HER2 antibodies could affect the existing FLS in high HER2 expressers. Specifically, we used trastuzumab (Tmab) and pertuzumab (Pmab) that respectively bind to domain IV (ref. 21) and II (ref. 22) of the HER2 ECD (extracellular domain). Additionally, we examined the effects of their fab fragments (Tfab and Pfab), to separate the consequences of the binding itself from other events that could result from antibody bivalency, such as receptor internalization23. HER2 distributions in the high expressers (MCF-7-HER2, SK-BR-3, BT-474, and KPL-4) were examined after 2 h and 1 day incubations with Tfab, Tmab, Pfab, and Pmab. Representative HER2 distributions from 1 day treatments in comparison to the untreated groups are shown in Fig. 3c for MCF-7-HER2 cells and in Supplementary Fig. 5a–d for

![Figure 4](https://example.com/figure4.png)

**Figure 4 | Membrane deformation occurs in HER2 3+ human breast tumours.** (a-c) Human benign breast epithelium (left), HER2 1+ (middle) and HER2 3+ breast ductal adenocarcinoma (right) are shown in (a) T-blue stained light microscopy (LM; Scale bars, 20 μm (left) and 5 μm (middle and right)), (b) HER2 IHC (scale bars, 40 μm) and (c) TEM images (scale bars, 0.5 μm (left and middle) and 2 μm (right)). The TEM images show representative cell morphologies. Tight junctions (cyan arrow), desmosomes (yellow arrows), well-defined cell interfaces (between two white dashed lines), and separation of cells (between two orange arrows) are identified. (d) TEM images of two different areas in the HER2 3+ sample. The image on the right is the magnified image of the area in the white rectangle in c. Some of the FLS and circular structures are indicated by green and violet arrows, respectively. Scale bars, 1 μm. (e) Fractions of deformed cells (mean ± s.e.m.) were assessed in a blinded manner. The specimens were from one normal (N), one adjacent normal (AN), four HER2 1+ (L1-L4) and three HER2 3+ (H1-H3) human tissue samples. The fraction was significantly higher in 3+ samples. ***P value < 10^-6 by two-sided, unpaired Welch’s t-test. EC, epithelial cell; ECM, extracellular matrix; Nuc, nucleus; TC, tumour cell.
all investigated cells. The distributions for the 2 h time points were similar (data not shown). In all high expressers, FLS densities were generally down-modulated by these four treatments. However, the relative effects of individual treatments varied among different high expressers. In Fig. 3c, HER2 distributions in MCF-7-HER2 after 1 day incubations with Tfab or Pfab appear more random and diffusive compared with those treated with Tmb or Pmab. On the other hand, in SK-BR-3 (Supplementary Fig. 5b,e), while Tfab clearly reduced FLS densities, the effect of Pfab was less obvious. Among the four high expressers, KPL-4 was least responsive to these treatments. FLS densities were obtained from cluster analysis and these are plotted in Fig. 3d for MCF-7-HER2 and SK-BR-3 (1 day treatments) and (Supplementary Fig. 5e) for all investigated cells (both 2 h and 1 day treatments). The fact that HER2 ECD binders can affect FLS at all, and also differently across various cell types is another indication that the membrane deformation results from physical interactions among HER2s, and also between the receptors and other features of the cell membrane, such as other membrane associated proteins or lipid microdomains. We suspected that a steric pressure generated from the ECDs of diffusing HER2s could be responsible, and tested this idea by overexpressing an ECD deletion mutant of HER2. Interestingly this ECD deletion mutant also induced membrane deformation (Supplementary Fig. 6a–c), suggesting the mechanism is more complex than an ECD crowding effect.

Membrane deformation appears in HER2 3+ human tumours. We next examined whether the extensive membrane deformation induced by HER2 overexpression observed in vitro also occurs in HER2 3+ human breast tumour cells. We examined cell membrane morphologies in breast tissue samples taken from 7 BC patients and one underwent reduction mammoplasty. These samples consisted of invasive ductal adenocarcinoma with HER2 overexpression (3+), and ductal adenocarcinoma with slightly elevated HER2 expression (1+). Benign breast epithelium (0) adjacent to a 3+ tumour region and normal breast epithelium (0). Figure 4a shows representative morphological profiles (bright-field microscopy images) of a benign breast epithelium (left), and HER2 1+ (middle) and 3+ (right) breast tumour samples. Both 1+ and 3+ tumour cells show cellular and nuclear pleomorphisms with enlarged nuclei and prominent nucleoli. Also shown are HER2 IHC images that indicate relative HER2 expression levels (Fig. 4b), and high-magnification TEM images (Fig. 4c) from each group. The adjacent benign breast epithelium (left) shows smooth cell membranes that exhibit apical-basolateral cell polarity with ducts, and tight junctions (cyan arrow) and desmosomes (yellow arrows) between cells. Interestingly tumour cells with just 1+ expression also show smooth membranes along with well-maintained cell–cell interfaces (white dashed lines; middle), while cell polarity is not apparent. In 3+ tumour cells, cell membranes are considerably deformed (right), epithelial polarity is not observed, and each cell is widely separated from neighbouring cells (orange arrows). Two magnified images (Fig. 4d) show deformed membrane morphologies in greater detail, including irregularly shaped and sized FLS (green arrows), and circular structures (violet arrow), as observed similarly in vitro. To establish statistics for HER2 expression-dependent membrane deformation phenotypes in human BC tissues, we counted the cells bearing both deformed membranes and perturbed cell–cell interfaces in 3–5 different regions of each tissue sample in a blinded manner (for details, see Supplementary Fig. 7a–d). The fractions of deformed cells (Fig. 4e) are significantly higher (>80%) in all three HER2 3+ breast tumour tissues than in four 1+ tumour samples (<20%) and adjacent normal (<4%) and normal breast tissues (none). These data clearly demonstrate that the membrane deformation is predominantly associated with HER2 3+ human breast tumour cells.

Membrane deformation reduces the cell contact area. The fact that most of the HER2 3+ cells with deformed membranes in the TEM images were in loose contact with each other suggests that these signalling-independent alterations of membrane morphology by high HER2 cell-surface densities may play a role in disease progression. Specifically, we speculated that the membrane deformation may reduce the surface area available to cells for interaction with either their surrounding substrates or neighbouring cells. In the former case, the membrane deformation would cause the area available for the formation of focal adhesion (FA) complexes to decrease. In the latter scenario the contact area eligible for forming cell–cell junctions would be reduced.

To address the reduced surface area with surrounding substrates, the distributions of FA complexes were compared with those of FLS by IF. We used integrin β1 (ITGB1) and paxillin to mark FA complexes, which are, respectively, an initiator of the complex assembly and an adaptor that can directly associate with the integrin. Indeed the IF images (Supplementary Fig. 8a) show little overlap between FA complexes and the elongated regions of high HER2 fluorescence signal (FLS) in MCF-7-wtHER2 cells. As expected, ~70% of the high-density regions of ITGB1 (blue) co-localize with paxillin (green; also shown in Supplementary Fig. 8b), while neither ITGB1 nor paxillin significantly overlaps with the high HER2 regions (red) in Fig. 5a. The reduced localizations were not due to a decrease in protein expression levels (Supplementary Fig. 8c). These results suggest the membrane deformation would reduce the cell-surface coverage of FA complexes.

To further validate the relationship between HER2 overexpression driven membrane deformation and reduced cell–substrate contact areas, we quantified how HER2 expression levels and receptor signalling activities in the stable cell lines affect the cell-surface coverage of paxillin. IF images for paxillin in MCF-7 stable transfectants are shown in Fig. 5b. In low expressers (MCF-7-vctrl and shH2-SK-BR-3), small paxillin clusters (~0.5 μm²) (ref. 29) densely covered the entire basal cell surfaces. However, in all high expressers of wtHER2 (MCF-7-wtHER2 and SK-BR-3) and the signalling-incompetent mutants (MCF-7-knHER2, and MCF-7-AicdHER2), as well as lapatinib treated SK-BR-3, the cell-surface densities of small paxillin clusters in the central regions were significantly reduced compared with low expressers. (See Supplementary Fig. 8d for IF images for paxillin in SK-BR-3 variants and Supplementary Fig. 8e for the comparison of the size-distributions of paxillin clusters between high and low HER2 expressers.) Similar results were obtained when MCF-7 cells transiently overexpressed wtHER2, knHER2, YFHER2 and AicdHER2 (see IF images of paxillin staining in these cells in Supplementary Fig. 8f). The ratios of the areas covered with paxillin relative to the total basal cell-surface area across the various cell types are compared in Fig. 5c. These results were independent of the substrates on which the cells were grown. We found the cell-surface coverage of paxillin was also reduced in high-expressor MCF-7-wtHER2 cells compared with MCF-7-vctrl cells, when these cells were grown on collagen (Supplementary Fig. 8g). The correlation between the reduced paxillin coverage and high HER2 expression, regardless of the receptor’s signalling activities, suggests that this reduction resulted from the HER2 overexpression induced membrane deformation.

Because FLS were observed on the entire cell surface of high HER2 expressers by TEM (Fig. 2d, and Supplementary...
Figs 3c and 6d), it is likely they may also perturb the formation of cell–cell contacts. To examine this, we visualized the locations of E-cadherin and ZO-1, which are present in adherens and tight junctions, respectively. In MCF-7 (low expresser), E-cadherin (green, Fig. 5d for stable transfectants) and ZO-1 (along with E-cadherin as shown in Supplementary Fig. 9a for transient transfectants) are clearly localized at the cell interfaces. This indicates the presence of solid cell–cell contacts. In contrast, for all high HER2 (red) expresser counterparts, regardless of receptor signalling capability, these localizations are greatly reduced (Fig. 5d and Supplementary Fig. 9a). Again this reduction was not caused by a decrease in the protein level.

**Figure 5 | The membrane deformation disrupts cell interfaces.** (a) Processed images of raw IF data (similar images are shown in Supplementary Fig. 7a,b) acquired from TIRFM imaging of HER2 (red, αH2Fab:QD), integrin beta 1 (ITGB1; blue, αCD29:alexa568) and paxillin (green, αpaxillin:Alexa488) in MCF-7-HER2 cells showing the relative locations of these proteins. Less overlap between HER2 and ITGB1 is observed than between ITGB1 and paxillin. Scale bars, 5 μm. (b) IF images of paxillin distributions in basal cell membranes of various MCF-7 transfectants. The Paxillin distributions in all high HER2 expressers (3+; wtHER2 and the two signalling-incompetent HER2 mutants) are irregular compared to the more uniform distribution in the low HER2 expresser MCF-7-vctrl. Scale bars, 20 μm. (c) Average cell-surface coverage (mean ± s.e.m.) of paxillin clusters (normalized (Nor.). Paxillin coverage) was calculated from the ratio of the area covered with paxillin (FLS greater than ~13% of the maximum intensity) to the total cell-surface area. ***P value < 10^-6 by two-sided, unpaired Welch’s t-test. (d) Confocal IF images of E-cadherin (green, αE-cadherin:alexa488) in various MCF-7 transfectants. Scale bars, 30 μm. (e) Confocal IF images of HER2 (red, αH2Fab:QD) and E-cadherin in MCF-7-wtHER2 (left panels) and MCF-7-ΔicdHER2 (right panels) co-cultured with MCF-7-vctrl to contrast the delocalized patterns of E-cadherin in high HER2 expressers (red arrows) with the localized E-cadherin distributions in low expressers (green arrows). Scale bars, 20 μm. (f) A cartoon illustrating how the HER2-induced membrane deformation perturbs cell–substrate and cell–cell interfaces, and thus disrupts epithelial integrity. Shades of beige and red represent different cells.
(Supplementary Fig. 8c). The disparate localization patterns of E-cadherin are clearly shown in the IF images of high expressers (wtHER2 and ΔicdHER2) co-cultured with MCF-vctrl on glass cover-slips (Fig. 5e) and in basement membrane extract matrices (Supplementary Fig. 9b; also see the relative fluorescence intensity profiles of HER2 vs E-cadherin cell-surface localizations). In these images, the adhesion protein is significantly more delocalized in the high expressers than in the low expressers. Importantly the reduced localizations were not caused by the loss of E-cadherin nor did they result in up-regulation of mesenchymal markers such as vimentin (see WB data in Supplementary Fig. 8c).

We performed a similar series of experiments on MCF10Dcis.com (DCis.com), a cell line originally derived from the MCF10A line, which forms predominantly comedo-type ductal carcinoma in situ (DCis) in vivo.31 DCIS is a non-invasive early-stage BC, but DCIS lesions with HER2 overexpression are likely to rapidly progress to invasive BC (ref. 14). As we expected, HER2 overexpression in DCis.com cells also reduced the cell-surface coverages of paxillin and E-cadherin (see IF data in Supplementary Fig. 9c). In vitro experiments, we consistently saw an inverse relationship between high HER2 surface expression and membrane localization of E-cadherin. All these data clearly demonstrate that cell interfaces can be perturbed by physical deformation of their membranes without transcriptional alterations that downregulate adhesion proteins.32–35 Additionally, the membrane deformation induced epithelial disruption are consistent with the morphological alterations of HER2 + 3 BCs observed in DCIS (refs 36,37).

Discussion

Most human BCs originate from epithelial cells and progress through multiple histological stages to become invasive and eventually spread to distant organs. This progression involves disruption of normal epithelial morphology and increased dissemination potential of tumour cells as a result of various genetic alterations.6,38-40. HER2 overexpression in BCs is known to facilitate this progression.4–7. Specifically HER2 overexpression that leads to disruption of epithelial integrity by causing a morphological deformation of cell membranes into FLS (summarized in Fig. 5f). These effects did not downregulate E-cadherin, which is implicated in invasive cancer progression by virtue of its ability to induce EMT (refs 12,32–35). Cell interface disruptions are known early-stage events in the EMT induced by HER2 overexpression in BC (ref. 12). In addition to the previously characterized effects of HER2 signalling on EMT, our data suggest that these interface disruptions may also result from signalling-independent, physical alterations of cell membranes by HER2 overexpression. This event is independent of, and orthogonal to, other well-described consequences of HER2 signalling activities.4,6–8,34. The membrane deformation we observed is not unique to HER2 overexpression as we found similar structures in cells overexpressing EGF.R. We hypothesize that this type of membrane alteration might represent an additional mechanism through which tumour cells overexpressing these receptor tyrosine kinases become less attached to their surroundings and more prone to acquiring an invasive phenotype.

Other types of membrane deformation have been associated with the pathogenesis of cancer. For example, invadopodia are actin-rich membrane structures that assist the invasiveness of tumour cells by recruiting matrix proteases to the tips of the protrusive structures and degrading the ECM (refs 41,42). Another membrane protrusion generated by cytoskeleton-regulatory proteins is called the filopodia-like protrusion, which is distinguished from filopodia by the ubiquitous distribution of ITGB1 and the long persistence of the protrusion. The integrin-rich filopodia-like protrusion becomes an assembly site for mature adhesions, and the establishment of adhesions in turn causes FAK-dependent mitogen-activated protein kinase/ERK (extracellular signal-regulated kinases) activation. This contributes to metastatic colonization of cancer cells in the post-extravasation stage.43. In contrast to these actin-mediated membrane protrusions, the FLS formations we observed were not dependent on the level of HER2 signalling nor were they strictly associated with actin. These observations suggest a different functional significance for this membrane morphological alteration.

We hypothesize FLS generation is mediated by physical processes, possibly involving elevated concentration of HER2 in lipid microdomains,44,45 which then leads to membrane bending.46–50 through protein–protein and protein–lipid interactions. This possibility is the subject of future investigation.

Methods

Mutagenesis and transfection. For transient transfection, wtHER2 (full length HER2) as well as knHER2 (K753M), Y7PHER2 (Y to F substitutions at Y877, Y1023, Y1139, Y1195, Y1221, Y1222 and Y1248), ΔicdHER2 (HER2 truncal domain truncation: 1-748aa), and AicdHER2 (HER2 extracellular domain truncation: 639-1255aa) genes that were fused with N-terminal gD epitope tags were cloned into plink5 expression vectors after site-directed mutagenesis. Primers used for each constructs are following.

knHER2: Forward: 5'-CCAGTGGCAGCATCTGTTTGAGGGA-3'; Y7PHER2: Forward: 5'-CATTGGCAGACGAGCATCCATCTGTTTGAGGGA-3'; ΔicdHER2: Forward: 5'-CCAGTGGCAGCATCTGTTTGAGGGA-3'; Y7PHER2: Forward: 5'-CTGCTGGGGTTAGGACACTCAGGTACACAGAG-3'; ΔicdHER2: Forward: 5'-GTGGCACGTCCAGACCCAGAAACTCTGGGTTCTCCACGGC-3'; Y7PHER2: Forward: 5'-GCCAGCTATAATTGTTGAGACAGCACTGAG-3'; ΔicdHER2: Forward: 5'-CTGCTGGGGTTAGGACACTCAGGTACACAGAG-3'; Y7PHER2: Forward: 5'-GCCCGCCAGCGTAATTTGTTGGAATCCAGGACTG-3'; ΔicdHER2: Forward: 5'-CTTCCTCCTGCGGTTGACAAAATCTCGGTTCTCGCAGGCC-3'; Y7PHER2: Forward: 5'-GCCAAGCTTCTGCAGGTC-3'; ΔicdHER2: Forward: 5'-GTCCTGGACGAGCTTTTCTGTTCGAGACGAC-3'; Y7PHER2: Forward: 5'-CTGGCACGTCCAGACCCAGAAACTCTGGGTTCTCCACGGC-3'; ΔicdHER2: Forward: 5'-GTCCTGGACGAGCTTTTCTGTTCGAGACGAC-3'; AicdHER2: Forward: 5'-GGACGTGCAGGAATTTTCTGAAGGCTTCACGAGAAGACGAC-3'; AicdHER2: Forward: 5'-GGACGTGCAGGAATTTTCTGAAGGCTTCACGAGAAGACGAC-3'; AicdHER2: Forward: 5'-GGACGTGCAGGAATTTTCTGAAGGCTTCACGAGAAGACGAC-3'; AicdHER2: Forward: 5'-GGACGTGCAGGAATTTTCTGAAGGCTTCACGAGAAGACGAC-3'; AicdHER2: Forward: 5'-GGACGTGCAGGAATTTTCTGAAGGCTTCACGAGAAGACGAC-3'; gD is derived from the HSV (herpes simplex virus) envelope glycoprotein D. MCF-7, SK-BR-3 and MCF10Dcis.com cells were transfected using FuGENE 6 Transfection Reagent (Promega), with the DNA constructs or siRNA oligos mixed in Opti-MEM (Life Technologies). Cells grown in 2D were incubated in the transfection mixture containing normal growth medium, RPMI (Roswell Park Memorial Institute) 1640 (MCF-7) and DMEM (SK-BR-3) with 10% foetal bovine serum (FBS) and 1% l-Glutamine, and DMEM/F12 50:50 (MCF10Dcis.com).
manufacturer (Invitrogen). Viral particles were harvested 48 h after transfection and MCF7 and MCF10 DCIS.com cells were infected. Cell pools expressing HER2 or HER2 mutants were selected using puromycin (Clontech Laboratories, 0.75 µg/ml -1 for MCF7-7 and 1.5 µg/ml -1 for DCIS.com transfectants).

**Cell-line culture.** Cell-lines used in this work were purchased from ATCC and DSMZ, and were maintained and authenticated using Short Tandem Repeat analysis by the Genotech central cell repository (gCell), which ensures that the cells were free of contamination. MCF-7, MCF-7-HER2, MDA-MB-231 XI.11, MDA-MB-453, EFM-192A and KPL-4 cells and maintained in RPMI 1640 with 10% FBS, and 1% -Glutamine in 5% CO2 at 37°C. MDA-MB-453, MDA-MB-175 VII and SK-Br-3 were kept in DMEM with 10% FBS, and 1% -Glutamine. For MCF10DCIS.com culture, DMEM/F12, with 5% horse serum, and 1% L-Glutamine was used. The condition was kept constant during the whole-imaging experiments and throughout cell preparation procedures for WB and IF. For imaging, cells were plated on glass-bottom dishes (MatTek; glass thickness: No. 1.5).

**FabQD conjugation.** Fab fragments of the HER2 antibody (7C2.B9 clone) were conjugated to QDs according to the protocol of the Qdot Antibody Conjugation Kit (Life Technologies), with minor modifications. 1.75 µl of a freshly dissolved 20 mM solution of sulfo-SMCC (Thermo Scientific) in DMSO was added to 62.5 µl (0.5 monole) of an 8 µM stock solution of amino-PEG-QD605 (Life Technologies). The mixture was incubated at room temperature (RT) for 1 h. In parallel, 100 µg of Fab was incubated in 0.2% PDS and reduced by adding 6.1 µl of 20 mM dithiothreitol/water solution at RT for 30 min. The sulfo-SMCC derivatized QDs were separated from excess unreacted sulfo-SMCC by passing the solutions over NAP-5 desalting columns (GE Healthcare) pre-equilibrated in 50 mM HEPES, 150 mM NaCl. Similarly, the reduced Fab fragments were passed over NAP-5 columns pre-equilibrated in the same HEPES/NaCl buffer to remove dithiothreitol. The derivatized QDs and reduced Fab fragments were mixed and allowed to react at RT for 2 h. Remaining free maleimide groups were deactivated by 2-mercaptoethanol (final concentration of 100 µM) at RT for 30 min. The FabQD conjugates were then concentrated by ultrafiltration (Pierce Protein Concentrator, Thermo Scientific), and separated from unconjugated Fab fragments by gel filtration (Superdex G200, GE Healthcare). The final conjugate pool in PBS was brought to 50% glycerol and kept at 4°C.

**Immuno-fluorescence.** Cells were maintained in their normal growth media at 37°C before treatment with Fixation and Permeabilization reagent (Life Technologies). Cells were incubated with primary antibodies for 3 h. For HER2 and ITGB1, primary antibodies that recognize the ECDs of these receptors were added to live cells before fixation. Bound antibodies were labelled with fluorescent secondary antibodies (Alexa 488/568 conjugated, Life Technologies), and imaged with TIRFM or confocal microscopy.

**Data availability.** The data that support the findings of this study are available upon request from the corresponding authors (I.C. and M.X.S.).

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Author contributions
I.C. conceived and designed the study, performed single molecule and imaging experiments and developed analyses methods, analysed EM data, performed mutagenesis and biochemical assays, evaluated stable cell lines and wrote the manuscript.
M.R. performed the EM experiments and evaluated the data; L.S. prepared viral constructs and created stable cell lines, and G.S. supervised LS’ work. R.W.A. developed QD-fab conjugates for single-molecule studies, H.K. and L.R. evaluated HER2 expression levels of tissue samples, M.X.S. and I.M. supervised the study, and wrote the manuscript.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications
Competing financial interests: M.R., L.S., R.W.A., H.K., L.R., I.M. and M.X.S. are full-time employees of Genentech, a member of the Roche Group, and have been granted options and own shares of Roche Holding AG.
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