HER2 expression identifies dynamic functional states within circulating breast cancer cells

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Circulating tumour cells in women with advanced oestrogen-receptor (ER)-positive/human epidermal growth factor receptor 2 (HER2)-negative breast cancer acquire a HER2-positive subpopulation after multiple courses of therapy1,2. In contrast to HER2-amplified primary breast cancer, which is highly sensitive to HER2-targeted therapy, the clinical significance of acquired HER2 heterogeneity during the evolution of metastatic breast cancer is unknown. Here we analyse circulating tumour cells from 19 women with ER-'/HER2- primary tumours, 84% of whom had acquired circulating tumour cells expressing HER2. Cultured circulating tumour cells maintain discrete HER2+ and HER2− subpopulations: HER2+ circulating tumour cells are more proliferative but not addicted to HER2, consistent with activation of multiple signalling pathways; HER2− circulating tumour cells show activation of Notch and DNA damage pathways, exhibiting resistance to cytotoxic chemotherapy, but sensitivity to Notch inhibition. HER2+ and HER2− circulating tumour cells interconvert spontaneously, with cells of one phenotype producing daughters of the opposite within four cell doublings. Although HER2+ and HER2− circulating tumour cells have comparable tumour initiating potential, differential proliferation favours the HER2+ state, while oxidative stress or cytotoxic chemotherapy enhances transition to the HER2− phenotype. Simultaneous treatment with paclitaxel and Notch inhibitors achieves sustained suppression of tumorigenesis in orthotopic circulating tumour cell-derived tumour models. Together, these results point to distinct yet interconverting phenotypes within patient-derived circulating tumour cells, contributing to progression of breast cancer and acquisition of drug resistance.

We documented the emergence of HER2+ circulating tumour cells (CTCs) in patients initially diagnosed with ER-positive/HER2-negative (ER+/HER2−) breast cancer, after multiple courses of therapy for recurrent metastatic breast cancer. Using microfluidic CTC-iChip purification followed by imaging flow cytometry3, 16 out of 19 (84%) patients had HER2+ CTCs (Fig. 1a, Extended Data Fig. 1a and Supplementary Table 1). Twenty-two individual CTCs from two representative patients (Brx-42, Brx-82) were isolated and subjected to single-cell RNA sequencing (scRNA-seq). HER2 expression was bimodal in distribution (≤1 read per million (RPM) versus median 133, range 32–217 RPM; P = 7.5 × 10−6) (Fig. 1b), indicating the existence of discrete HER2+ and HER2− subpopulations. In these patients, the fraction of HER2+ CTCs increased with disease progression (Extended Data Fig. 1b). HER2+ CTCs were not restricted to ER+/HER2− breast cancer; 2 out of 13 patients with ER−/PR+/HER2− (triple negative) breast cancer also had HER2+ and HER2− CTC subpopulations (Extended Data Fig. 1c). In ER+/HER2− breast cancers, immunohistochemical (IHC) staining of patient-matched metastatic tumour biopsies showed increased HER2+ staining, compared with primary tumours (Fig. 1c). Unlike HER2-amplified breast cancer, HER2+ tumour cells within metastatic lesions did not have evidence of gene amplification (Extended Data Fig. 1d).

The CTC-iChip efficiently captures viable CTCs, enabling derivation of CTC cultures4. We established CTC lines (Brx-42, Brx-82, Brx-142) with discrete HER2+/HER2− subpopulations comparable to patient-matched primary CTCs (Fig. 1a, d and Extended Data Fig. 1e, f). Acquired HER2 expression was not due to gene amplification, and no distinguishing mutations were identified between HER2+ and HER2− subpopulations (Extended Data Fig. 1g and Supplementary Table 2). Fluorescence-activated cell sorting (FACS) of HER2+ versus HER2− subpopulations showed distinct functional properties: HER2+ CTCs had a higher proliferation rate (Fig. 1e), with increased staining for the proliferation marker Ki67, but no change in apoptotic markers cleaved-caspase 3 or annexin 5 (Extended Data Fig. 2a, b).

We tested the relative tumorigenicity of HER2+ versus HER2− CTCs following injection into the mouse mammary fat pad. Both FACS-purified HER2+ and HER2− CTCs generated tumours, with HER2+ tumours being larger and having a higher frequency of lung metastases (Fig. 1f and Extended Data Fig. 2c, d). Despite differences in proliferation, limiting dilution studies showed that HER2+ and HER2− CTCs initiate tumours from as few as 200 cells, pointing to comparable progenitor potential (Extended Data Fig. 2e).

The coexistence of HER2+ and HER2− CTCs, despite differing proliferation rates, led us to test whether these subpopulations are capable of interconversion. After 4 weeks in culture, FACS-purified green fluorescent protein (GFP)-tagged HER2+ CTCs acquired HER2− cells (Brx-82: 42%; Brx-142: 46%), while HER2+ CTCs generated HER2− cells at lower efficiency (Brx-82: 5%; Brx-142: 11%) (Fig. 2a, b and Extended Data Fig. 3a). By 8 weeks, the parental HER2+/HER2− composition was nearly re-established (Fig. 2b). This interconversion was also evident by mixing equal proportions of GFP−/HER2− cells, with the emergence of GFP+/HER2+ and GFP+/HER2− CTCs, with the emergence of GFP+/HER2+ and GFP+/HER2− cells, respectively (Extended Data Fig. 3a).

To better define the timing of HER2−/HER2+ interconversion, we established single-cell-derived CTC colonies using HER2-based FACS, followed by sequential confocal microscopy. Colonies were scored for HER2 and EpCam expression at 1-, 3-, 5- to 9-, 10- to 19- and >20-cell stages. Single HER2− CTCs initially proliferated slowly (Extended Data Fig. 3b), and first acquired HER2+ daughter cells at the 5- to 9-cell stage (6.5%), with rapid interconversion thereafter (10–19 cells: 47%, >20 cells: 59%; Fig. 2c, d). The more rapidly proliferating single HER2+ CTCs also generated HER2+ progeny at the 5- to 9-cell stage (5%), but the proportion of HER2−/HER2+ CTCs rose more slowly

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Figure 1 | Distinct properties of HER2+ and HER2− CTC subpopulations from patients with advanced ER/HER2 breast cancer. a, Quantitation by imaging flow cytometry of HER2+ and HER2− CTCs isolated from patients Brx-42, Brx-82, EpCAM (yellow) and HER2 (green). Scale bar, 10 μm. b, Bimodal distribution of ERRB2 RNA-seq reads from single CTCs, Hartigan’s dip test, $P = 7.5 \times 10^{-6}$, $n = 22$ (HER2 $\leq 1$ RPM; HER2$ > 133$, range 32–217). c, IHC for HER2 (brown) in matched metastatic versus primary tumours (Brx-42, Brx-82, Brx-142) compared with HER2-amplified tumour (control). Scale bar, 100 μm; tumour data (Supplementary Table 1). d, FACS of cultured CTCs, showing discrete HER2+ and HER2− subpopulations. MDA-231 (triple-negative breast cancer (TNBC)) and SKBR3 (HER2-amplified) cells are shown as control. Scale bar, 100 μm.}

(10–19 cells: 17%, >20 cells: 22%; Fig. 2c, d). Thus, interconversion between HER2+/HER2− phenotypes occurs spontaneously as early as four cell doublings.

Interconversion between HER2+ and HER2− phenotypes was also tested in vivo by orthotopic inoculation of FACS-purified cultured CTCs. Tumours established from HER2− CTCs displayed HER2+ subpopulations, and vice versa (Fig. 2e and Extended Data Fig. 2c).

In vivo interconversion was confirmed by injecting a 1:1 mixture of GFP+/HER2+ and GFP−/HER2− CTCs (or the converse), followed by dual GFP and HER2 IHC. Within mixed tumours, GFP-tagged HER2− CTCs produced GFP+/HER2+ cells (44%), and in separate tumours, GFP-tagged HER2+ CTCs generated GFP+/HER2− cells (21%) (Fig. 2f and Extended Data Fig. 3d).

To define the molecular characteristics of HER2+ versus HER2− CTCs, we quantitatively mapped the global proteomes (>6,300 proteins) of FACS-purified subpopulations (Brx-42, Brx-82, Brx-142) using multiplex mass spectrometry (MS) with isobaric tandem mass tags (TMT) (Supplementary Table 3). While proteome profiles of individual cell lines were distinct, they shared differences between HER2+ and HER2− subpopulations (Pearson correlation coefficients: Brx-82 versus Brx-142 = 0.81; Brx-82 versus Brx-42 = 0.71; Brx-42 versus Brx-142 = 0.64) (Fig. 2a and Extended Data Fig. 4a, b). HER2+ CTCs showed enrichment (Pathway Interaction Database (PID)) of receptor tyrosine kinase (RTK) and pro-growth signalling (GSEA, false discovery rate (FDR) ≤ 0.25) (Fig. 2b and Supplementary Tables 3 and 4). Phosphoryrosine blots from the HER2+ subpopulations confirmed RTK phosphorylation (HER2, HER3, HER4, insulin receptor (INSR), EPHA1, EPHA2 and EPHA10), which was absent from matched HER2− CTCs.
HER2+ CTCs (Extended Data Fig. 4c). scRNA-seq analysis of 15 primary HER2+ CTCs compared with 7 HER2− CTCs from matched patient blood samples showed enrichment for 15 of 32 shared pathways (ERBB1, ERBB2/ERBB3, IGFI, EPHA2, MET) identified by MS analysis of cultured CTC lines (Fig. 3b, Extended Data Fig. 4d, e and Supplementary Tables 4 and 5). In contrast to HER2+ CTCs, MS analysis of cultured HER2− CTCs showed increased expression of proteins enriched in Notch (HES/HEY, Presenilin 1 (PS1)) and DNA damage pathways (AuroraB, ATM, ATR, Fanconi) (GSEA, FDR ≤ 0.25; nominal P cut-off < 0.05; Supplementary Table 4). Coloured shapes represent proteins within denoted pathways. Red asterisks highlight RTK pathways in b, and Notch pathways in c.

HER2+ CTCs (Extended Data Fig. 4c). scRNA-seq analysis of 15 primary HER2+ CTCs compared with 7 HER2− CTCs from matched patient blood samples showed enrichment for 15 of 32 shared pathways (ERBB1, ERBB2/ERBB3, IGFI, EPHA2, MET) identified by MS analysis of cultured CTC lines (Fig. 3b, Extended Data Fig. 4d, e and Supplementary Tables 4 and 5). In contrast to HER2+ CTCs, MS analysis of cultured HER2− CTCs showed increased expression of proteins enriched in Notch (HES/HEY, Presenilin 1 (PS1)) and DNA damage pathways (AuroraB, ATM, ATR, Fanconi) (GSEA, FDR ≤ 0.25; nominal P cut-off < 0.05; Supplementary Table 4). Coloured shapes represent proteins within denoted pathways. Red asterisks highlight RTK pathways in b, and Notch pathways in c.

To explore the potential therapeutic significance of pathways differentially activated in HER2+ versus HER2− CTC subpopulations, we screened a panel of 55 drugs selected both for clinical relevance and for the ability to target MS-identified pathways (Supplementary Table 6). HER2+ CTCs were no more sensitive to the HER2 inhibitor lapatinib than HER2− CTCs (half-maximum inhibitory concentration (IC50) = 1 μM), indicating they were not ‘oncogene addicted’ to HER2, unlike the HER2-amplified SKBR3 cells (IC50 = 5 nM) (Fig. 4a, Extended Data Fig. 5a, b). However, dual inhibition of HER2 and IGFI1R, another RTK activated in HER2+ CTCs, was cytotoxic to HER2+ but not HER2− CTCs (Fig. 4a), suggesting inhibition of multiple receptor tyrosine kinases may be effective in treating HER2+ CTCs. Compared with HER2+ CTCs, HER2− CTCs showed reduced sensitivity to the chemotherapeutic agents docetaxel, doxorubicin and 5-fluorouracil (5-FU) (Fig. 4b and Extended Data Fig. 5a, c), but increased sensitivity to γ-secretase inhibitors, which suppress Notch activity (Fig. 4b and Extended Data Fig. 5a, d). Despite proteomic enrichment for Aurora B signalling, HER2− CTCs were not differentially sensitive to Aurora family inhibitors (Fig. 3c, Extended Data Fig. 5a and Supplementary Tables 3 and 4).

The increased NOTCH1 in HER2− CTCs observed by quantitative MS and confirmed by western blot (Extended Data Fig. 6a and Supplementary Tables 3 and 4) was inversely correlated with HER2 expression within primary CTCs and CTC lines, shown by scRNA-seq and immunostaining (Extended Data Fig. 6a). We therefore tested the consequences of suppressing HER2 or activating Notch signalling in HER2+ CTCs.

Manipulation of NOTCH1 or its downstream effector NFE2L2/NRF2 in cultured HER2+ CTCs did not reduce HER2 expression (Extended Data Fig. 6b). However, inhibition of HER2 using lapatinib or short interfering RNA (siRNA) led to increased expression of NOTCH1, its ligands JAG1 and DLL1, and Notch-regulated genes HES1, HEY1 and HEY2 (Fig. 4c and Extended Data Fig. 6c), confirming previous reports from HER2-amplified breast cancer cells.67 (Extended Data Fig. 6c). Suppression of HER2 also resulted in increased expression of genes (GCLC, GGT1, GPX1, GPX4, HMOX1) downstream of Notch-regulated NRF2, a transcriptional regulator of anti-oxidant/glutathione metabolism pathways.68 (Extended Data Fig. 6d). Thus, expression of HER2 in CTCs appears to mediate downregulation of the NOTCH1/NRF2 axis, potentially switching between proliferative and survival-prone phenotypes.

In addition to suppressing HER2 directly, we tested additional stimuli capable of modulating the HER2+/HER2− interconversion. Treatment of HER2+ CTCs with low doses of docetaxel (1 nM) or induction of oxidative stress with hydrogen peroxide (H2O2; 10 mM) induced rapid shifts from HER2+ to HER2− (30% conversion, > 70% survival) (Fig. 4d). To exclude differential cell death, we demonstrated acceleration in the appearance of HER2− progeny from FACS-purified single HER2+ CTCs (5- to 9-cell stage: 45%; > 10-cell stage: 62%) (Fig. 4e and Extended Data Fig. 6e). Thus, exposure to cytotoxic/oxidative stress mediates a switch to a less proliferative but more drug-resistant phenotype.

To model the potential significance of HER2+/HER2− interconversion in vivo, we generated orthotopic mammary xenografts from FACS-purified subpopulations and analysed tumours before and after treatment with paclitaxel. Purified HER2+ CTCs generated mixed tumours (88% HER2+, 12% HER2−) and showed dramatic tumour shrinkage following paclitaxel treatment. The recurrent tumour showed a transient reduction in HER2+ with a corresponding increase in HER2− composition following chemotherapy (2 weeks: 39% HER2+; 7 weeks: 74% HER2+; Fig. 4f). Purified HER2+ CTCs also gave rise to a mixed tumour (35% HER2+, 65% HER2−), but paclitaxel induced only a limited delay in tumour growth with a minimal effect on HER2 content. Shedding of CTCs was also suppressed by paclitaxel in HER2+ but not HER2− tumours (Extended Data Fig. 6f). The chemotherapy-induced shift in HER2 composition was also evident following inoculation of parental CTC cultures (untreated 65% HER2+; post-therapy 30% HER2+; Extended Data Fig. 6g). Finally, we generated tumours from a 1:1 mixture of GFP-tagged HER2+ and untagged HER2− cells, demonstrating a shift from GFP+/HER2+ to GFP−/HER2− cells following paclitaxel treatment (untreated: 70% GFP+/HER2+, post-therapy: 42% GFP+/HER2−; Extended Data Fig. 6h). The potent effect of chemotherapy on HER2+/HER2− phenotypes in vivo may reflect both reduced drug-sensitivity of HER2− cells, as well as stress-induced HER2+ to HER2− switching.

Given the demonstrated susceptibility of HER2− CTCs to Notch inhibitors, we combined paclitaxel with either of two γ-secretase

Figure 3 | Molecular pathways differentially activated in HER2− versus HER2+ cultured CTCs. a, Comparison of quantitative MS proteomes (6,349 proteins) showing distinct profiles for individual cultured CTCs (BxR-82, BxR-142), but linear correlation between proteins differentially expressed in HER2− and HER2+ subpopulations; Nl, normalized intensity; n = 2 biological replicates per CTC line BxR-42, BxR-82, BxR-142 (Supplementary Table 3). b, c, Cytoscape network maps (top) and GSEA pathway analysis (bottom) depicting proteins enriched by greater than log2(0.5) by quantitative MS in (b) HER2+ and (c) HER2− CTCs (GSEA FDR ≤ 0.25; nominal P cut-off < 0.05; Supplementary Table 4). Coloured shapes represent proteins within denoted pathways. Red asterisks highlight RTK pathways in b, and Notch pathways in c.
**Figure 4 | Cooperative targeting of HER2+ and HER2− CTC subpopulations suppresses tumour growth.**

a, HER2+ CTCs show no change in sensitivity to lapatinib alone, compared with matched HER2− CTCs (Brx-142), but have increased sensitivity to combined HER2 and IGF1R (BMS-754807) inhibitors; n = 6; s.d. (error bar). b, HER2+ CTCs demonstrate reduced chemosensitivity (docetaxel) but have enhanced sensitivity to Notch inhibition (BMS-708163, Notchi1), compared with HER2− CTCs; n = 6; s.d. (error bar). c, Inhibition of HER2 with lapatinib or siRNA-mediated knockdown in HER2+ cells with loss of HER2 at indicated time points. Scale bar, 10 μm. d, Rapid emergence (96 h) of HER2+ CTCs following treatment of HER2+ CTCs with H2O2 (10 mM) or docetaxel (1 nM). e, Confocal microscopy showing rapid appearance of HER2− progeny from single-CTC derived HER2+ colonies treated with H2O2. EpCAM (green), HER2 (red) and MERGED (gold). Scale bar, 20 μm; n = 10. Arrows and dashed boxes indicate cells with loss of HER2 at indicated time points. f, Paclitaxel treatment (4 weeks) of mice with CTC-derived (Brx-142) orthotopic mammary tumours. Top: HER2+/HER2− tumour growth curves with paclitaxel treatment; bottom: representative IHC for HER2 (brown) in HER2+ and HER2− derived tumours at the U (untreated), T (2 weeks post-treatment) and R (7 weeks post-treatment) time points. Scale bar, 100 μm; t-test, *P < 0.05, ****P < 0.0001. g, Simultaneous treatment (4 weeks) of mammary xenografts (Brx-82) with paclitaxel and either Notch inhibitor RO4929097 (Notchi2) or LY-411575 (Notchi3), showing sustained responses for the combination, compared with paclitaxel alone. Rx denotes treatment duration; two-way ANOVA, P < 0.0001, n = 6.

Inhibitors (LY-411575; RO4929097) in treating mice with tumours initiated from parental CTC lines. Compared with paclitaxel alone, the combination therapy significantly delayed onset of tumour recurrence, while Notch inhibition alone had no effect on tumour growth (Fig. 4g and Extended Data Fig. 6i).

Taken together, we have used primary and cultured CTCs from patients with ER+/HER2− breast cancer who developed metastatic multidrug-resistant disease to show that coexisting distinct HER2+ and HER2− tumour cell subpopulations may interconvert, with striking consequences for disease progression and drug response. The comparable tumour initiating potential and similar expression of stem cell marker ALDH1 in HER2+ and HER2− CTCs suggest underlying tumour cell plasticity in these advanced patient-derived breast CTC lines, rather than a hierarchical cancer stem-cell model as described in drug-resistant subpopulations within established breast cancer cell lines16–18. While expression of NOTCH1 and other embryonic markers has been reported in rare, quiescent cells within primary breast tumours16–18, the NOTCH1+ CTCs reported here constitute a major...
cell population, exhibiting both persistent cell proliferation in vitro and tumorigenesis in vivo. Thus, we propose a dynamic model, in which the equilibrium between HER2+ and HER2− cells within a heterogeneous tumour population is driven by spontaneous interconversion between these phenotypes, with the more rapidly proliferating HER2+ cells prevalent under baseline conditions, and environmental or therapy-induced stress enhancing conversion to the more resistant HER2− phenotype. Neither molecular profiling nor functional studies have revealed secreted factors that affect the mutual survival of HER2+ and HER2− CTCs, but we cannot exclude such additional factors.

Finally, the properties of patient-derived CTC lines established after multiple courses of therapy provide relevant insight to the treatment of drug-refractory, advanced breast cancer. While clinical trials are evaluating the efficacy of HER2-targeted therapy in HER2+ breast cancer with acquired HER2+ CTCs,19–21, our observations indicate that acquisition of HER2 does not indicate HER2 oncogene dependence and drug susceptibility; instead it constitutes a marker of a proliferative, multi-RTK state. Furthermore, the interconversion of chemotherapy-sensitive HER2+/NOTCH1+ and NOTCH inhibitor-sensitive HER2−/NOTCH1− CTCs suggests that dual treatment, as modelled here, may be required for effective treatment. Clinical trials so far have had limited success sequentially administering embryonic pathway inhibitors targeting Hedgehog, Wnt or Notch to inhibit cancer stem cells following initial chemotherapy.6,22–25. The rapid interconversion between proliferative and drug-resistant CTC subpopulations raises the possibility that simultaneous combination therapy may provide a novel strategy for clinical validation.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper. References unique to the Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to the Supplementary Information are available in the online version of the paper. for clinical validation.

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1. Arteaga, C. L. & Engelman, J. A. ERBB receptors: from oncogene discovery to basic science to mechanism-based cancer therapeutics. Cancer Cell 25, 282–303 (2014).

2. Houssami, N., Macaskill, P., Balleine, R. L., Bilous, M. & Pegram, M. D. HER2 discordance between primary breast cancer and its paired metastasis: tumor biology or test artefact? Insights through meta-analysis. Breast Cancer Res. Treat. 129, 659–671 (2011).

3. Özkumur, E. et al. Inertial focusing for tumor antigen-dependent and -independent sorting of rare circulating tumor cells. Sci. Transl. Med. 5, 179ra7 (2013).

4. Yu, M. et al. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. Science 345, 216–220 (2014).

5. Ting, L., Rad, R., Gygi, S. P. & Haas, W. MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. Nature Methods 8, 937–940 (2011).

6. Osipo, C. et al. ErbB-2 inhibition activates Notch-1 and sensitizes breast cancer cells to a γ-secretase inhibitor. Oncogene 27, 5019–5032 (2008).

7. Abdolvand, A. & Edgerton, M. L. Notch promotes recurrence of dormant tumor cells following HER2/neu-targeted therapy. J. Clin. Invest. 125, 2484–2496 (2015).

8. DeNicola, G. M. et al. Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. Nature 475, 106–109 (2011).

9. Nakabayashi, N. et al. Notch-Nrf2 axis: regulation of Nrf2 gene expression and cytotoxicity by notch signaling. Mol. Cell. Biol. 34, 653–663 (2014).

10. Korkaya, H. & Wicha, M. S. HER-2, notch, and breast cancer stem cells: targeting an axis of evil. Clin. Cancer Res. 15, 1845–1847 (2009).

11. Ihimakin, S. et al. HER2 drives luminal breast cancer stem cells in the absence of HER2 amplification: implications for efficacy of adjuvant trastuzumab. Cancer Res. 73, 1635–1646 (2013).

12. Korkaya, H., Paulson, A., lovino, F. & Wicha, M. S. HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. Oncogene 27, 6120–6130 (2008).

13. Gineo, C. et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 1, 555–567 (2007).

14. Martz, C. A. et al. Systemic identification of signaling pathways with potential to confer anticancer drug resistance. Sci. Signal. 7, ra121 (2014).

15. Pandya, K. et al. Targeting both Notch and ErbB-2 signalling pathways is required for prevention of ErbB-2-positive breast tumour recurrence. Br. J. Cancer 105, 796–806 (2011).

16. Takeke, N., Harris, P. J., Warren, R. Q. & Ivy, S. P. Targeting cancer stem cells by inhibiting Wnt, Notch, and Hedgehog pathways. Nature Rev. Clin. Oncol. 8, 97–106 (2011).

17. Vanharanta, S. & Massague, J. Origins of metastatic traits. Cancer Cell 24, 410–421 (2013).

18. Lawson, D. A. et al. Single-cell analysis reveals a stem-cell program in human metastatic breast cancer cells. Nature 526, 131–135 (2015).

19. Bidard, F. C. & Perga, J. Y. Clinical utility of circulating tumor cells in metastatic breast cancer. J. Clin. Oncol. 33, 1622 (2015).

20. Schramm, A. et al. The DETECT Study Program: personalized treatment in advanced breast cancer based on circulating tumor cells (CTCs). ASCO Meet. Abstr. 33, TPS11095 (2015).

21. Ignatiadis, M. et al. Abstract OT1-2-02: trastuzumab in HER2-negative early breast cancer as adjuvant treatment for circulating tumor cells (CTC) (Treat CTC). Cancer Res. 75, OT1–2–02 (2015).

22. Amakye, D., Jagani, Z. & Dorsch, M. Unraveling the therapeutic potential of the Hedgehog pathway in cancer. Nature Med. 19, 1410–1422 (2013).

23. Kim, E. J. et al. Pilot clinical trial of hedgehog pathway inhibitor GDC-0449 (vismodegib) in combination with gemcitabine in patients with metastatic pancreatic adenocarcinoma. Clin. Cancer Res. 20, 5937–5945 (2014).

24. Lo Russo, P. M. et al. Phase I trial of hedgehog pathway inhibitor vismodegib (GDC-0449) in patients with previously advanced or metastatic solid tumors. Clin. Cancer Res. 17, 2502–2511 (2011).

25. Krop, I. et al. Phase I pharmacologic and pharmacodynamic study of the gamma secretase (Notch) inhibitor MK-0752 in adult patients with advanced solid tumors. J. Clin. Oncol. 30, 2307–2313 (2012).

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Author Contributions N.V.J., D.A.H. and S.M. conceived the project and provided project leadership. A.B. enrolled patients and provided clinical guidance. B.S.W. and S.R. performed the bioinformatics analyses, M.L. and Y.Z. assisted with animal experiments. T.K.S., M.L.O. and A.J.J. performed the mutational analysis and fluorescence in situ hybridization, J.A.L., R.O. and R.E. performed the bioinformatics analyses. M.L. and Y.Z. assisted with molecular biology experiments. D.S. analysed pathology specimens. C.B. and M.Y. helped with expert flow cytometry. B.S.W. and S.R. performed the bioinformatics analyses. M.L. and Y.Z. assisted with molecular biology experiments. D.S. analysed pathology specimens. C.B. and M.Y. assisted with animal experiments. T.K.S., M.L.O. and A.J.J. performed the mutational analysis and fluorescence in situ hybridization, J.A.L., R.O. and R.E. performed the bioinformatics analyses. M.L. and Y.Z. assisted with molecular biology experiments. D.S. analysed pathology specimens. C.B. and M.Y. assisted. B.S.W. and S.R. performed the bioinformatics analyses. M.L. and Y.Z. assisted with molecular biology experiments. D.S. analysed pathology specimens. C.B. and M.Y. assisted with animal experiments. T.K.S., M.L.O. and A.J.J. performed the mutational analysis and fluorescence in situ hybridization, J.A.L., R.O. and R.E. performed the bioinformatics analyses. M.L. and Y.Z. assisted with molecular biology experiments. D.S. analysed pathology specimens. C.B. and M.Y. assisted. B.S.W. and S.R. performed the bioinformatics analyses. M.L. and Y.Z. assisted with molecular biology experiments. D.S. analysed pathology specimens. C.B. and M.Y. assisted.
CTC culture. CTC cultures were grown in suspension in ultra-low attachment plates (Corning) in tumour sphere medium (RPMI-1640, EGF (20 ng/ml), bFGF (20 ng/ml), 1x B27, 1x antibiotic/antimycotic (Life Technologies)) under hypoxic (4%) conditions. The breast CTC lines, Brx-42, Brx-82 and Brx-142, were derived from CTCs isolated using the CTC-iChip as previously described4. CTC lines were routinely checked for mycoplasma, using a mycoplasma detection kit (MycoAlert, Lonza), and were authenticated by RNA-sequencing and DNA-sequencing (1,000 gene mutation panel).

Fluorescence activated cell sorting (FACS). Cells were trypsinized into single-cell suspensions, resuspended in Hank’s balanced solution (HBSS), and incubated with Anti-HER2/NEU APC (BD, clone 42-c-erbB-2), Anti-HER2 FITC (Janssen R & D) or Annexin V FITC (BD, clone RUO) antibodies for 20 min at 4°C. Unbound antibodies were washed from cells using HBSS. For analytical flow, cells were fixed with 3% paraformaldehyde and analysed using a Laser BD Fortessa instrument. For sterile live-cell flow cytometry, cells were sorted using a Laser BD FACS Aria Fusion Cell Sorter, BSL2+. FACS plots are representative of at least two independent experiments performed within 6 months of culture initiation (Figs 1d and 2a and Extended Data Figs 1f and 3a).

Sequence analysis of genomic DNA. Genomic DNA extracted from CTC-derived cell lines was sequenced using a multiplex polymerase chain reaction (PCR) technology called Anchored Multiplex PCR (AMP) for single nucleotide variant (SNV) and insertion/deletion (indel) detection using next generation sequencing (NGS) as previously described33. Briefly, genomic DNA was isolated from cell lines and then sheared with the Covaris M220 instrument, followed by end-repair, adenylation and ligation with an adaptor. A sequencing library targeting hotspots and exons in 39 commonly mutated, cancer-associated genes was generated using two hemi-nested PCR reactions. Illumina MiSeq 2 × 151 base paired-end sequencing results were aligned to the hg19 human genome reference using RNA MEM31. MuTect32 and a laboratory-developed insertion/deletion analysis algorithm were used for SNV and indel variant detection, respectively. This assay has been validated to detect SNV and indel variants at 5% allelic frequency or higher in target regions with sufficient read coverage.

Lentivirous production, infection and siRNA knockdown of CTC cell lines. To produce replication-incompetent lentiviruses, 293T cells were co-transfected with either Lenti-Luc-GFP or Notch intracellular domain-pcw107 (Addgene 64621) constructs in combination with REV, VS VG, PDM L or Pmd2.G and psPAX2 (Addgene) using Lipofectamine Plus reagent (Invitrogen). Twenty-four hours later, growth medium was replenished. Viral supernatants were harvested 48 h post-transfection, concentrated with Lenti-X Clonect (Clontech), and viral pellets were resuspended in 400 μl base medium. CTC cultures were infected overnight with 100 μl lentiviruses in 6 μg/ml Polybrene. Puromycin (3 μg/ml) was used to select transduced cells over a period of 7 days. For the RNAi knockdown, CTC lines Brx-42, Brx-82 and Brx-142 were reverse transfected in ultra-low attachment six-well plates (Corning) with 25 μM siRNA smart pools (Dharmacon) containing the combination of four different siRNA oligonucleotides for ERBB2/ HER2 (GGACGAAUUCUCAGCAUG; GACGAAUUCUGCAACAUU; CGAACAACAGACGCAUUG; AGCCGAAAUUGACUGAUG), HER2 (GGCGACGUGUGACGUGU1; GAUGCCGAAUGACGUGU2; GACAGGCCGACUCAACAGAU; GAAGGGGACUAACACUGGA; NRE2L2 (GAGAAAGAUAUGCCUGUAA; CCAAACAGCAGACGUAUG; UAAGAUGGUCUGUCAGAUA); the negative control gene GAPDH. siRNA pools for target genes were deconvolved to demonstrate targeted knockdown efficiency (more than two siRNAs per gene).

Immunofluorescence. CTC lines were spun onto poly-L-lysine-functionalized glass slides with Spintrap, fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained with primary antibodies against EpCAM (Abcam, clone H597), Epcam (Cell Signaling Technology, clone VU19D) and HER2 (Cell Signaling Technology, clone 29D8) antibodies.

CTCs were isolated from fresh whole blood by depleting leukocytes using the microfluidic CTC-iChip as previously described. Briefly, whole blood samples were incubated with biotinylated antibodies against CD45 (R&D Systems, clone 2D1), CD66b (AbD Serotec, clone 80H3) and CD16 (BD, clone 3G8) followed by incubation with Dynabeads MyOne Streptavidin T1 (Invitrogen) to achieve magnetic labelling of white blood cells. This mixture was processed through the CTC-iChip, and the CTCs were stained in solution with Alexa 488-conjugated antibodies against EpCAM (Cell Signaling Technology, clone VU19D) and HER2 (Cell Signaling Technology, clone 29D8 or Janssen R&D) and identified by imaging flow cytometry (Aminis). Individual CTCs were picked after staining as described above, and PE-CF594-conjugated antibody against CD45 (BD Biosciences, clone HJ30) was included to stain contaminating leukocytes. CTCs were individually micromanipulated using a 10-μm transfer tip on an Eppendorf TransferMan NK 2 micromanipulator, transferred into PCR tubes containing RNA protective lysis buffer, and flash frozen in liquid nitrogen as previously described29. Standard CTC enumeration of fixed samples is performed on the BioView high content imaging system following Megafunnel fixation and staining with the combination of wide spectrum cytokeratin (Abcam, clone 8E10), Epcam (Cell Signaling Technology, clone VU19D), EGFR (Cell Signaling Technology, clone D38B1) and HER2 (Cell Signaling Technology, clone 29D8) antibodies.

For mouse xenograft studies, blood was collected via cardiac puncture and ~1 ml of blood was processed through the microfluidic CTC-iChip. CTCs were enumerated on the BioView imaging system after staining with Alexa 488-conjugated antibodies against EpCAM (Cell Signaling Technology, clone VU19D), HER2 (Janssen R&D or Cell Signaling Technology, clone 29D8) and GFP (ab13970) followed by secondary antibodies conjugated with Alexa-488 (Invitrogen).

Immunohistochemistry. Tissues were sectioned, and slides were incubated in 0.3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase activity. Tissues were permeabilized, and antigen retrieval was performed in 1× citrate buffer (pH 6) for 15 min. Slides were washed and blocked for 30 min with 5% goat serum. Primary HER2 (Cell Signaling Technology, 29D8) or GFP (Living Colours AV 632381) antibodies were diluted 1:75 or 1:250 in DAKO antibody diluent and samples were incubated for 1 h at room temperature. Slides were incubated with HRP anti-rabbit antibody (EnVision + DAKO) for 30 min. After washing with PBS, the peroxidase reaction was performed with 3.3′-diaminobenzidine (DAB) from Vector Laboratories for 10 min. Cells were counterstained with Gill’s #2 haematoxylin for 10–15 s, dehydrated with ethanol and cleared with xylene before mounting. Images represent at least five independent fields from six to eight xenograft tumours per condition.

Fluorescence in situ hybridization. Fluorescence in situ hybridization was performed as described previously27,28. Briefly, 5-μm sections of formalin-fixed, paraffin-embedded tumour samples were de-paraffinized, hydrated and pretreated with 0.1% pepsin for 1–2 h. Slides were then washed in 2× saline-sodium citrate buffer (SSC), dehydrated, air dried and co-denatured at 80°C for 5 min with a mixture of CEP17 and HER2 probes and hybridized at 40°C overnight using the Hybride Hybridization System (Abbott). Two-minute post-hybridization washes were performed at 2× SSC/0.3% NP40 at 72°C followed by a 1 min wash in 2× SSC at room temperature. Slides were mounted with Vectashield containing 4′,6-diamidino-2-phenylindole (Vector, Burlingame, California, USA). Entire sections were observed with an Olympus BX61 fluorescent microscope equipped with a charge-coupled device camera and analysed with Cytovision software (Applied Imaging, Santa Clara, California).

The HER2 and CEP17 signals were quantified in 50 randomly selected, non-overlapping nuclei, and mean numbers of HER2 and CEP17 copies per nucleus were calculated. HER2 was specified amplified when the HER2/CEP17 ratio was >2.0 or HER2 signals per nuclei was >6 following the guidelines of the American Society of Clinical Oncology/College of American Pathologists36. The probes used in this study consisted of centromeric CEP 17p11.1-q11.1, spectrum aqua (Abbott Molecular, Des Plaines, Illinois) and locus-specific identifier probes derived from bacterial artificial chromosome RP11-94L15 (17q12-17q21.1, spectrum orange probe (CHORI, Oakland, California)).

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Determination of reads-per-million (RPM). Trimomatic was used to crop reads to 50 nucleotides, and to remove the TruSeq3-PE-2 Illumina adapters. The paired-end reads were then aligned using tophat2 and bowtie1 with the no-misjunction argument set with human genome version hg19 and transcriptome defined by the hg19 genes.gtf table from http://genome.ucsc.edu. Reads that did not align or aligned to multiple locations were discarded. The number of reads aligning to each gene was then determined using htseq-count. Samples that had fewer than 106 reads were discarded. The read count for each gene was divided by the total counts assigned to all genes and multiplied by one million to form the reads per million (RPM). Samples for which the expression of the white blood cell marker PTPRC (CD45) was greater than 10 RPM were discarded. Single-cell RNA-seq data have been deposited in the Gene Expression Omnibus under accession number GSE75367.

Bimodality. To establish the distribution of HER2 expression in CTCs is multi-modal, we applied the Hartigans’ dip test as implemented in the diptest R-package to the log_{10}(RPM + 1) values with 10 RPM as the threshold to define HER2− versus HER2+ CTCs. To establish that the distribution has two modes and not more, we applied the density function of R with default values to the log_{10}(RPM + 1) values.

Gene set enrichment analysis of RNA-seq and quantitative proteomics data. On the basis of the analysis of bimodality above, we defined HER2+ samples to be those for which the expression of HER2 exceeded 10 RPM and defined the rest to be HER2−. For the mass spectrometric data, enrichment of signalling pathways was determined by submitting the average log, fold-change in protein abundance between the HER2-high and HER2-low samples to the pre-ranked function of the Broad Institute’s GSEA software using gene sets in the Pathway Interaction Database (PID) and KEGG as curated in version 4 of the Broad Institute’s MSigDB (http://www.broadinstitute.org/gsea/msigdb/). Pathway enrichment for the RNA-seq of the CTCs and the HER2+ versus HER2− distinction was input to the GSEA software instead of log, fold-change.

Quantitative proteomics. CTC cell pellets were re-suspended in lysis buffer containing 75 mM NaCl, 50 mM HEPES (pH 8.5), 10 mM sodium pyrophosphate, 10 mM NaF, 10 mM β-glycerophosphate, 10 mM sodium orthovanadate, 10 mM phenylmethylsulfonylfluoride, Roche Complete Protease Inhibitor EDTA-free tablets and 3% sodium dodecyl sulfate. Cells were lysed by passing them ten times through a 21-gauge needle, and the lysates were prepared for analysis on the mass spectrometer essentially as described previously3. Briefly, reduction and thiol alkylation were followed by purifying the proteins using MeOH/CHCl3 precipitation. Protein digest was performed with Lys-C and trypsin, and peptides were labelled with TMT-10plex reagents (Thermo Scientific)34 and fractionated by basic pH reversed phase chromatography. Multiplexed quantitative proteomics was performed on an Orbitrap Fusion mass spectrometer (Thermo Scientific) using a simultaneous precursor selection (SPS)-based MS3 method34. MS2 spectra were assigned using a SEQUEST-based proteomics analysis platform35. On the basis of the target−decoy database search strategy36 and employing linear discriminant analysis and posterior error histogram sorting, peptide and protein assignments were filtered to a FDR of < 1% (ref. 35). Peptides with sequences that were contained in more than one protein sequence from the UniProt database were assigned to the protein with most matching peptides35. TMT reporter ion intensities were extracted as that of the most intense ion within a 0.03- Thomson window around the target–decoy database search strategy36 and employing linear discriminant analysis and posterior error histogram sorting, peptide and protein assignments were filtered to a FDR of < 1% (ref. 35). Peptides with sequences that were contained in more than one protein sequence from the UniProt database were assigned to the protein with most matching peptides35. TMT reporter ion isotopologues with isobaric masses. Protein interactions were extracted from the STRING database (high confidence score > 0.7)37. Overlapping proteins were assigned to the pathway with the greatest number of proteins, and enriched PID pathways were ranked by log_{10}(P-value) to the nearest thousandth. Mass spectrometry raw data have been deposited in the MassIVE proteomics data repository under the accession number MSV00079419.

Drug screens. Drugs were obtained from the MGH Center for Molecular Therapeutics and are listed in Supplementary Table 6. They were chosen because of their common clinical use for treatment of breast cancer or unique targeting of epigenetic/stem cell pathways. One thousand cells were seeded in tumour sphere media in 384-well ultra-low attachment plates in triplicate wells on duplicate plates 24h before the addition of drugs. Three independent drug concentrations centred on the reported IC_{50} were used (Supplementary Table 6). Cell viability was assayed 6 days after drug treatment with CellTiter-Glo (Promega) and was normalized to corresponding untreated controls38.

Mouse xenograft assays and drug treatment. In compliance with ethical regulations and approved by the animal protocol (IACUC 2010N00006), 6-week-old female NSG (NOD. Cg-Pkscnsdd1I12rtgim1Wjl/Szjax) mice from Jackson Laboratories were anaesthetized with isofluorane, and GFP-LUC labelled CTCs (200,000, 20,000 and/or limiting dilutions as low as 200 cells) or 50:50 mixed CTCs (GFP–LUC+/HER2−: Untagged/HER2+, and the converse) were injected into the fourth right mammary fat pad. A 90-day release 0.72 mg oestrogen pellet (Innovative Research of America) was implanted subcutaneously behind the neck of each mouse. Tumour growth was monitored weekly by in vivo imaging using IVIS Lumina II (PerkinElmer) following intraperitoneal injection (150 μg per animal) of α-luciferin substrate (Sigma). For in vivo drug sensitivity testing, Paclitaxel (10 mg/kg) was administrated weekly by intravenous injection for 4 consecutive weeks. Notch inhibitors (Notch3) L1-411575 (10 mg/kg) or (Notch1) RO429097 (10 mg/kg) were administrated daily (5 days on/2 days off) via oral gavage in 2% solvent (2% sodium carboxymethyl cellulose) for 4 consecutive weeks. No animal randomization or blinding was used for these mouse studies. All animal studies used six to eight mice per condition to ensure sufficient statistical power.

26. Miyamoto, D. T. et al. RNA-Seq of single prostate CTCs implicates noncanonical Wnt signaling in antiandrogen resistance. Science 349, 1345–1356 (2015).
27. Mohapatra, G. et al. Gicoma test array for use with formalin-fixed, paraffin-embedded tissue: array comparative genomic hybridization correlates with loss of heterozygosity and fluorescence in situ hybridization. J. Mol. Diagn. 8, 268–276 (2006).
28. Snuderl, M. et al. Polysomy for chromosomes 1 and 19 predicts earlier recurrence in anaplastic oligodendrogliomas with concurrent 1p/19q loss. Clin. Cancer Res. 15, 6430–6437 (2009).
29. Wolff, A. C. et al. American Society of Clinical Oncology/Collage of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J. Clin. Oncol. 25, 118–145 (2007).
30. Zheng, Z. et al. Anchored multiplex PCR for targeted next-generation sequencing. Nature Med. 20, 1479–1484 (2014).
31. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754–1760 (2009).
32. Cilibuski, K. et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nature Biotechnol. 31, 213–219 (2013).
33. McAlister, G. C. et al. Increasing the multiplexing capacity of TMs using reporter ion isotopologues with isobaric masses. Anal. Chem. 84, 7469–7478 (2012).
34. McAlister, G. C. et al. MultiNotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. Anal. Chem. 86, 7150–7158 (2014).
35. Huttlin, E. L. et al. A tissue-specific atlas of mouse protein phosphorylation and expression. Cell 143, 1174–1189 (2010).
36. Elias, J. E. & Gygi, S. P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nature Methods 4, 207–214 (2007).
37. Szklarczyk, D. et al. The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. Nucleic Acids Res. 39, D561–D568 (2011).
38. Garnett, M. J. et al. Systematic identification of genomic markers of drug sensitivity in cancer cells. Nature 483, 570–575 (2012).
Extended Data Figure 1 | See next page for caption.
Extended Data Figure 1 | Patients with advanced ER+/HER2− breast cancer harbour discrete HER2+ and HER2− subpopulations.

a, CTCs freshly isolated from 19 patients with ER+/HER2− breast cancer were stained with HER2 (green) and EpCAM (yellow) and imaged using imaging flow cytometry. Bar graph shows the number of HER2+ (black) and HER2− (white) CTCs (median 22% HER2+ CTCs, range 4–58%). Supplementary Table 1 provides HER2+/HER2− ratios and each patient’s clinical history. b, scRNA-seq for ERBB2 expression at multiple time-points showing acquisition of HER2+ CTCs (Brx-82, Brx-42) over the course of progressive disease. Single asterisk (*) denotes patient expiration. Rx, sacituzumab (IMMU-132); Rx1, vinorelbine + trastuzumab; Rx2, eribulin. c, Distinct HER2+ and HER2− CTCs from 13 patients with triple-negative breast cancer (TNBC) determined by scRNA-seq (HER2− ≤ 0 RPM; HER2+ > 153, range 33–463). d, HER2 fluorescence in situ hybridization (FISH) analysis of metastatic tumours from patients, Brx-42, Brx-82 and Brx-142, shows no amplification of ERBB2 compared with HER2-amplified control (Supplementary Table 1 for tumour source data). HER2 (red); chromosome enumeration probe 17 (CEP17) (cyan); scale bar, 10 μm. Representative images from five independent fields are shown. e, Bright field and immunofluorescence (DAPI, blue; HER2, green) images of CTC lines, Brx-42, Brx-82 and Brx-142, demonstrate heterogeneity in HER2 expression. Scale bar, 100 μm (bright field); 20 μm (immunofluorescence). Representative images from three independent fields are shown. f, FACS analysis shows two distinct HER2+ and HER2− subpopulations in the CTC line Brx-42 (at initiation) compared with HER2− control. Representative data of two independent experiments are shown. g, HER2 FISH analysis of the HER2+ and HER2− subpopulations from CTC lines Brx-42, Brx-82 and Brx-142 shows that ERBB2 is not amplified. HER2-amplified SKBR3 cells shown as control. HER2 (red); CEP17 (green); scale bar, 10 μm. Representative images from five independent fields are shown.
Extended Data Figure 2 | HER2+ and HER2− subpopulations exhibit distinct functional properties. a, Increased expression of the proliferation marker Ki67 (red) in the HER2+ subpopulation of CTC line Brx-142 (t-test, P < 0.0001), compared with the HER2− subpopulation, with no change in cleaved-caspase 3 (red). HER2+ cells (green); scale bar, 20 μm. Representative images from five independent fields are shown. b, FACS analysis for the apoptotic marker Annexin V-FITC shows no difference in apoptosis between the HER2+ and HER2− subpopulations of FACS-purified CTC line Brx-142. Representative data from two independent experiments are shown. c, Tumours initiated by HER2+ or HER2− CTCs (Brx-82: 200,000 cells) orthotopically injected into the mammary fat pad show differential growth rates; n = 8. d, Metastatic frequency of HER2+ and HER2− cultured CTCs (Brx-82: P = 0.05; Brx-142: P = 0.009) following orthotopic injection; n = 8. e, Limiting dilution experiments demonstrate comparable tumour initiating ability from 200 HER2+ and HER2− cultured CTCs (Brx-82, Brx-142); n = 8.
Extended Data Figure 3 | Dynamics of HER2+ and HER2− interconversion. a, FACS-purified HER2+ and HER2− subpopulations from CTC line Brx-82 were monitored over 28 days to determine shifts in the composition of sorted populations. Representative data of two independent experiments are shown. b, Growth curves for HER2+ (red) and HER2− (blue) FACS-purified single cell clones from CTC line Brx-142; two-way ANOVA, P < 0.0001; n = 20. c, IHC HER2 staining of tumour xenografts derived from unlabelled HER2− and HER2+ CTCs showing acquisition/loss of HER2 (brown), respectively. Arrows indicate regions of HER2 acquisition/loss. Representative image from at least five independent fields; n = 8. ER+/HER2− and HER2-amplified breast cancers are shown below as controls. d, Low-magnification (landscape) view of HER2 IHC staining of tumour xenografts derived from mixed HER2+ and HER2− CTC cultures containing either GFP-tagged HER2+/HER2− cells (high magnification images are shown in Fig. 2f). Top: representative GFP-tagged HER2− cells give rise to GFP+/HER2− cells (GFP: cytoplasmic red stain, HER2: cell surface brown stain). Bottom: GFP-tagged HER2+ cells produce GFP+/HER2− cells. Scale bar, 100 μm.
Extended Data Figure 4 | Proteomic and scRNA-seq analysis of HER2⁺ versus HER2⁻ cells. a, b, MS-based whole cell proteome profiles (6,349 proteins) comparing HER2⁺ and HER2⁻ populations from CTC lines (Brx-42, Brx-82, Brx-142). Matched HER2⁺ versus HER2⁻ proteomic differences show significant linear correlation (Pearson correlation coefficient = 0.71 between Brx-82 and Brx-42; Pearson correlation coefficient = 0.64 between Brx-142 and Brx-42); NI, normalized intensity; n = 2 per cell line are shown. c, Phospho-RTK array of HER2⁺ and HER2⁻ populations of CTC cell lines Brx-142 and Brx-82 show increased phosphorylation of RTKs in the HER2⁺ population. Numbers denote the following: 1, HER2; 2, HER3; 3, HER4; 4, INSR; 5, EPHA1; 6, EPHA2; 7, EPHA10. Representative data from two independent experiments are shown. d, Volcano plot depicts genes enriched in HER2⁺ (red) and HER2⁻ (blue) individual CTCs isolated from patients Brx-42 and Brx-82 and analysed by scRNA-seq; n = 22. e, Venn diagram showing PID pathway overlap of genes and proteins derived from scRNA-seq (Brx-42, Brx-82) and quantitative proteomics of HER2⁺ CTCs, respectively.
Extended Data Figure 5 | Fifty-five panel drug screen shows differential drug sensitivities exhibited by HER2+ versus HER2− subpopulations. a, Heat map showing percentage cell viability (represented as decimal) after 6 days of drug treatment of the HER2+ and HER2− subpopulations derived from CTC lines Brx-142 and Brx-82. Red and blue represent high and low drug sensitivities, respectively; n = 6. b, Lapatinib sensitivity of HER2+ (red) and HER2− (blue) subpopulations of CTC line Brx-82. MDA-231 (TNBC) and SKBR3 (HER2-amplified) are shown as controls. c, Chemosensitivity of HER2+ (red) and HER2− (blue) subpopulations of CTC line Brx-142 to Notch inhibition with Notch1 (BMS-708163) and Notch2 (RO4929997). MDA-231 and SKBR3 cells are shown as controls. a–d, Representative of at least two independent experiments for each condition; n = 6.
Extended Data Figure 6 | See next page for caption.
Extended Data Figure 6 | NOTCH1 expression and activity in HER2−CTCs. a, Western blot analysis of HER2+ and HER2−subpopulations from CTC lines Brx-142 and Brx-82 show increased NOTCH1 in HER2−cells. β-Actin is shown as control. Immunofluorescence analysis and scRNA-seq of NOTCH1 (red) and HER2 (green) shows inversely correlated expression in CTC lines (Brx-142, Brx-82). b, Ectopic expression of constitutively active Notch intracellular domain (ICD) or NRF2 results in increased expression of the Notch1 ligand JAG1 but does not alter HER2 expression. Representative data of two independent experiments are shown; s.e.m. (error bars). c, siRNA-mediated inhibition of HER2 in Brx-42 HER2+CTCs, and lapatinib-mediated inhibition of HER2 in SKBR3 cells results in dose-dependent increases in the expression of genes involved in Notch signalling (NOTCH1, JAG1, DLL1, HES1, HEY1, HEY2). Representative data of two independent experiments are shown; s.e.m. (error bars). d, Inhibition of HER2 using lapatinib or siRNA knockdown in Brx-82 HER2+ CTCS increases the expression of NRF2-driven cytoprotective genes downstream of the Notch pathway. Representative data of two independent experiments are shown; s.e.m. (error bars). e, Quantitation of the interconversion of HER2+ cells from single-cell clones into 5- to 9-cell and >10-cell clusters following treatment with 10mM H2O2; t-test, P < 0.05; n = 10. f, Paclitaxel treatment of mice with tumours derived from Brx-142 FACS-purified HER2+CTCs, demonstrating a reduction in CTCs, and HER2−CTCs with no change in counts; t-test P < 0.05; NS, not significant. g, Paclitaxel treatment of mice with mammary xenografts derived from parental CTC line Brx-142 showing initial tumour response, followed by recurrent tumour growth. IHC analysis and quantitation of the recurrent tumour shows greatly reduced HER2+ (brown stain) cell composition in the Paclitaxel drug treated (T, 3 weeks post-treatment) tumour compared with the untreated tumour U, and the recovered tumour (R, 5 weeks post-treatment). Bar indicates duration of drug treatment (Rx). Scale bar, 100 μm; two-way ANOVA, P < 0.0001; n = 6. Representative images from five independent fields per tumour are shown and quantified; t-test, P < 0.001. h, Dual GFP (red, cytoplasmic stain) and HER2 (brown, cell surface stain) IHC of tumour xenografts derived from mixed GFP-tagged HER2+ and untagged HER2−CTC cultures demonstrating enhanced conversion from GFP+/HER2+ to GFP−/HER2−after 4 weeks of paclitaxel treatment; t-test, P < 0.0001; n = 6. Scale bar, 100 μm. Arrows indicate interconverting cells. Representative images from five independent fields per tumour are shown. i, Mouse tumour xenografts derived from the CTC line Brx-142 treated with a combination of the Notchi3 (LY-414575) and paclitaxel shows diminished tumour relapse; n = 6. Bar indicates treatment duration.