METHODS

Pooled shRNA screening

MCF-10A and MCF-10A/ErbB2* cells were infected with a pool of virus generated from the Open Biosystem GIPZ lentiviral human shRNA library (Silva et al, 2005) that comprises 58,493 shRNA targeting 18,651 genes. After infection, cells were cultured in triplicate in standard plastic plates for 10 doubling times or embedded in ECM-Matrigel for one week. In addition, MCF-10A/ErbB2* cells were injected in triplicate into the intra mammary fat pad (i.m.f.p.) of immunocompromised mice to perform the in vivo screening. Genomic DNA was extracted from the T0 and the final time point using Qiagen blood and cell culture DNA kit (Genomic Tip 500/G) following the manufacturer’s instructions. shRNA sequence was amplified by PCR using FastStart Taq polymerase using the following primers:

Fw1 5’- AATGATACGGGCACCAGATCTACACTTTCCCTACACGACGCTTTCGATCTatacgTAGTGAAGCCACAGATGTA -3’
Fw2 5’- AATGATACCGAGCCACGAGATCTACACTTTCCCTACACGACGCTTTCGATCTcgatgtTAGTGAAGCCACAGATGTA -3’
Fw3 5’- AATGATACCGGCACCAGATCTACACTTTCCCTACACGACGCTTTCGATCTttaggcTAGTGAAGCCACAGATGTA -3’
Fw4 5’- AATGATACGGGCACCAGATCTACACTTTCCCTACACGACGCTTTCGATCTtgaccaTAGTGAAGCCACAGATGTA -3’
Fw5 5’- AATGATACGGGCACCAGATCTACACTTTCCCTACACGACGCTTTCGATCTacagtgTAGTGAAGCCACAGATGTA -3’
Fw6 5’- AATGATACGGGCACCAGATCTACACTTTCCCTACACGACGCTTTCGATCTggcaatTAGTGAAGCCACAGATGTA -3’
Rv1 5’- CAAGCAGAAGACGGCATAACAGCTCTTCCGATCTGTAATCTGATTGTCCA -3’

Both microarray (barcode-probed and hairpin-probed, Open BioSystem) and NGS sequencing (Illumina HiSeq) technologies were used to quantify shRNA abundance. Experimental details were described in the book chapter (Rodriguez-Barrueco et al., 2013).

Processing of shRNA screening data

Microarray-based shRNA screening data was background corrected by negative control probes. Signals of duplicate probes were averaged. For hairpin-based array data, we averaged sense and anti-sense probe signals for the same hairpin. The processed data
was normalized by quantile algorithm. We processed NGS-based screening data by counting 22bp hairpin reads uniquely mapped to a hairpin sequence in the library. Raw count data was scale-normalized to 10M total reads per sample and log2-transformation. Due to small sample size, we also controlled the variance of hairpin representation data in each triplicate to be less than the 80% quantile of variance in the entire library. Computational and QC details were described in the book chapter (Yu et al., 2013).

**Differential representation analysis of shRNA screens**

We used a Bayesian linear model (Gelman et al., 2004) to estimate individual shRNA dropout effects in MCF-10A/ErbB2* vs. WT cells. To estimate the gene-level effects of a gene targeted by multiple shRNAs, we applied a Bayesian Hierarchal modeling approach (Gelman et al., 2004). This model allowed “random effects” from different shRNAs, and a coefficient of ‘fixed effects’ was used to score capability of increasing sensitivity at gene level. Bayesian-MCMC computing was set up for accurate estimations.

**Meta-analysis of shRNA dropouts**

To generate robust dropout candidates in MCF-10A/ErbB2* cells from shRNA screening data, we combined evidence from different sources, e.g., microarray data and NGS data under 2D, 3D or in vivo systems. We performed this meta-analysis using Stouffer’s z score method (Stouffer et al., 1949) shown in the following formula.

\[
Z = \frac{\sum_{i=1}^{k} z_i}{\sqrt{k}}, \quad z_i \sim N(0,1)
\]

In the above equation, \( z_i \) is the z-score indicating the strength of evidence, for example, differential representation score of a gene or a hairpin, in one source, designated number i from total number of k sources. \( z_i \) follows a standard normal distribution, so the integrated Z score also follows a standard Gaussian distribution assuming independence of all k evidence. Combined two-tailed p value was calculated based on the integrated Z score.

**Data-driven network re-construction of breast cancer**

We used a data-driven approach, ARACNe (Margolin et al., 2006) to reconstruct a breast cancer interactome from 359 TCGA breast cancer gene expression profiles. We
then applied the ARACNe algorithm against 1597 probes corresponding to 780 TFs to establish a TF-centered interactome and against 6434 probes for 2453 signaling molecule genes to construct a signaling protein-focused network. The parameters of the algorithm were configured as follows: p-value threshold \( p = 1 \times 10^{-7} \), DPI tolerance \( e = 0 \), and number of bootstraps \( NB = 100 \). We used the adaptive partitioning algorithm for mutual information estimation.

**Gene expression microarray data processing**

We processed TCGA microarray data (Agilent G4502A platform) of 359 breast cancer primary samples using loess normalization (Futschik and Crompton, 2004) and filtered the dataset to 24,401 probe sets with non-specific filtering. The microarray data (Illumina HT12v3) of MCF-10A/ErbB2* experiments was normalized by VSN (Huber et al., 2002) and RSN (Du et al., 2008) methods. METABRIC gene expression data was downloaded from synapse of Sage Bionetwork (Bionetworks).

**Gene expression arrays**

For gene expression arrays MCF-10A and MCF-10A/ErbB2* RNA was extracted using RNeasy extraction kits and labeled using the Low Input QuickAmp Labeling Kit (Agilent #5190-2331) following manufacturer’s instructions. Finally, labeled RNAs were hybridized on Illumina HT12v3 Microarray. The experiment was performed with an \( n=6 \).

MCF-10A cells were treated with IL6 (50\( \mu \)g/ml) for 1 hour. In parallel MCF-10A/ErbB2* infected with inducible shRNAs against STAT3 were incubated with doxyxycycline (100ng/ml) for 5 days. RNA from each condition was extracted with RNeasy extraction kit, labelled and hybridized on Human GE 4x44K v2 Microarray Kit (Agilent # G4845A).

Gene expression data has been uploaded and the GEO accession number assigned is: GSE62251

**Differential-expression analysis**

To overcome small sample size problem of expression profiles and to produce robust signature genes, we used a Probit regression model (Gelman et al., 2004) for differential-expression analysis. Bayesian-MCMC computing was employed to estimate
parameters for its robustness and accuracy. In particular, a t-distribution prior and Gibbs sampling were used in this analysis (Gelman et al., 2008).

**Master regulator analysis**

We interrogated the TCGA breast cancer interactomes and applied the MARINa algorithm (Carro et al., 2010; Lefebvre et al., 2010; Piovan et al., 2013) to identify key master transcriptional or signaling drivers for MCF-10A/ErbB2* cells or HR-/HER2+ breast cancer samples in TCGA or METABRIC data. For GSEA method in MARINa algorithm, we applied ‘maxmean’ statistic (Efron and Tibshirani, 2007) to score the enrichment of the gene set and used sample permutation to build the null distribution for statistical significance.

**Patient databases**

Patient data were assessed from cBioPortal (http://www.cbioportal.org/public-portal/), Metabric (Curtis et al., 2012), and TCGA (TCGA, 2012; http://cancergenome.nih.gov/)

**Viral Production and Infection**

Lentiviral production was achieved by transfecting phoentix-packaging cells with jet-PEI (Polyplus #101-10N) in combination with the lentiviral plasmids (shRNA listed below, pLOC_S100A8, pLX304-Blast-V5_STAT3) and the pCMV-dR8.91 and pMD.G helper plasmids at a ratio of 2:1:1 respectively (Rodriguez-Barrueco et al, 2013). The same conditions were employed to produce retroviral particles by combining the retroviral plasmids (pBABE-HERTVMA, pLPCX-RFP_S100A9) with the retroviral helper plasmids Psi and VSVg. Twenty four hours after transfection, packaging cells were cultured with regular MCF-10A growing media for 24 hours; afterwards, the media containing the viral particles was collected and concentrated.

Cells were plated at 60% confluence in a 6-well plate; and after 24 hours, cells were cultured in normal media mixed with media containing the viral particles (ratio 1:1). ,

Cells were reinfected 12 hours later, following the same procedure. Infecting media was replaced after 12 hours with fresh media and the cells were selected with the appropriate antibiotic.

Lentiviral shRNA used were:

| Gene name | Clone ID       | Vector | Accession #    |
|-----------|----------------|--------|----------------|
| STAT3     | V2LHS_88502    | pGIPZ  | NM_00315       |
STAT3      V2LHS_262105      pGIPZ      NM_00315
STAT3      V2THS_88502      pTRIPZ      NM_00315
STAT3      V2THS_26210      pTRIPZ      NM_00315
AGRN       V3LHS_330987      pGIPZ      NM_198576
AGRN       V3LHS_330988      pGIPZ      NM_198576
AGRN       V3LHS_330989      pGIPZ      NM_198576
GLRX       V2LHS_25191      pGIPZ      NM_002064
GLRX       V3LHS_410184      pGIPZ      NM_002064
S100A8     V2THS_31839      pTRIPZ      NM_002964
S100A9     V2THS_377130      pTRIPZ      NM_053587

**Western Blotting**

Cells were washed with cold PBS and lysed with EZ lysis buffer (1M Tris pH7, 50% glycerol, 20% SDS, 1mM ortovanadate, 1mM sodium fluoride and 1mM phenylmethylsulfonyl fluoride). Protein concentrations were determined by the Protein Assay Kit (Bio-Rad #500-0006). Equal amounts of proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare #10401197). Non-specific binding was blocked by incubation with TBST (20 mM Tris-Hcl pH7.4, 150 mM NaCl, 0.1% Tween-20) plus 5% of non-fat milk. Membranes were incubated with the primary antibodies overnight at 4°C and for 1 hour with secondary HRP-conjugated antibodies at room temperature (Amersham #NA9350V, #NA931V and #NA934V). Signal was detected with Lumi-Light Western Blotting Substrate (Roche #12015200001 and #12015196001).

The antibodies used in this study include pErbB2 (Tyr1221/1222) (Cell Signaling #2243), ErbB2 (Calbiochem#OP15), pSTAT1 (Tyr701) (Cell Slgning #9171), pSTAT (Tyr705) (Cell Signaling #9145), pSTAT5 (Tyr694) (Cell Sigaling #9359), STAT1 (Cell Signaling #9172), STAT3 (Cell Signaling #4904), STAT5 (Cell Signaling #9363), S100A8 (R&D #MAB4570), S100A9 (Santa Cruz #sc-20173),pAkt (Thr308) (Cell Signaling #2965), Akt (Cell Signaling #4691), pErb1/2 (Thr202) (Cell Signaling #4370), Erk1 (Santa Cruz #sc-94), p-p70S6K (Thr389) (Cell Signaling #9234), p70S6K (Cell Signaling #9202), MYC (Santa Cruz #sc-40 and sc-42), CLND1 (BD #556470), E1A (Calbiochem #DP11), p53 (Cell Signaling #9282), β-Actin (USBiological #A0760-40).
**SOC3, S100A8, S100A9 promoter cloning and luciferase assay**

To measure luciferase activity, phoenix cells were plated at 70% confluence in 96-well plates. Twenty-four hours later cells were transfected with 50ng of pGL3 constructs containing the promoter sequences in combination with a Renilla normalization control using jet-PEI transfection reagent. After 24 hours relative luciferase units (RLU) were measured using the Dual-Glo Luciferase Assay System (Promega #E2949).

Promoter sequences were amplified from MCF-10A genomic DNA by using the following primers:

**SOCS3**

| Fw  | 5'- AAAAA ACG CTG GAA CCG GGA GGC TCT CCA GGT -3' |
|-----|--------------------------------------------------|
| Rv  | 5' AAAAA AAG CTT GGC GCA CGG AGC CAG CGT GGA -3' |

**S100A8**

| Fw  | 5'- AAAAA ACG CTG GAT CAA GCA AGT GGA TGC -3' |
|-----|------------------------------------------------|
| Rv  | 5' AAAAA CTC GAG GCC CAC GGA CTT GCC CCA -3'    |

**S100A9**

| Fw  | 5'- AAAAA ACG CTG AAC AAA TAT TTG TTG CAT AAA -3' |
|-----|--------------------------------------------------|
| Rv  | 5' AAAAA CTC GAG CTG CTT GCA CTC TGT CTG TGT -3' |

**RNA extraction, Reverse Transcription and Real-time PCR**

RNA from cell lines was extracted using RNeasy extraction Kit following the manufacturer's recommendations (QIAGEN #74106). 0.5 micrograms of total RNA was converted into cDNA using the High Capacity cDNA Reverse Transcription Kit (Roche #4368814) according to manufacturer's instructions. The Real-time PCR reaction was performed with 10uL FastStart SYBR Green Master Mix (Roche #04673492001), 2 uL of complementary DNA (cDNA) and 1uL of each primer in RNase-free water adjusted to 20uL volume reaction. The thermal cycler conditions were as follows: AmpliTaq activation 95ºC for 3 minutes, denaturation 95ºC for 10 seconds, and annealing/extension 60ºC for 30 seconds (repeat 40 times). Triplicate Ct values were further analyzed ($2^{-\Delta\DeltaCT}$) by normalizing to an endogenous reference gene (GAPDH or Rn18s). Results are presented as the relative mRNA amount compared to the untreated samples.

Primers for human genes used:

**GAPDH**

| Fw  | 5'- CATCTTCTTTTGCGTCGC -3' |
|-----|-----------------------------|
| Rv  | 5' AAAAGCAGCCCTGTTGAC -3'   |

**S100A8**

| Fw  | 5'- ATGCCGTCTACAGGGATGAC -3' |
|-----|------------------------------|
| Rv  | 5' ACGCCCATCTTTATCACCAG -3'  |

**S100A9**

| Fw  | 5'- TCATCAACACCTTCCACCAA -3' |
SOCS3  Fw: 5’- CCTGCGCCTCAAGACCTTC -3’
Rv: 5’- GTGTCAGGTCTCCATGAT -3’

STAT3  Fw: 5’- AGTATAGCCGCTTCCTGCAA -3’
Rv: 5’- GTCACTGCGCTCCAGTAG -3’

AGRN  Fw: 5’- ATGCTCAACTCCAGCCCTCAT -3’
Rv: 5’- GCAATCTCCATTGGCTTCTC -3’

GLRX  Fw: 5’- TCGATATCAGCCACCAAC -3’
Rv: 5’- CACTGCATCCGCTATACAA -3’

**Competition assay**

MCF-10A or MCF-10A/ErbB2* were mixed with equal amounts of their corresponding variant (2.5 x 10^4 cells each) in 6-well plates. The fluorescent signal (GFP or RFP) co-expressed with the shRNA was monitored by FACS every three days and represented as a line graph.

**Soft agar colony formation assay**

Cells were plated in semi-solid media as follows: 35mm plates were layered with 0.6% agar (Spectrum Chemical, AG110) and media, 5.0 x 10^3 cells ml^-1 were seeded in triplicate in the second layer of 0.3% agar and media, and cultured in appropriate growth media. Colonies were stained with 1mg/ml MTT (Sigma, M2128) after three weeks of incubation, photographed, counted, and the average ± standard deviation was represented.

**Three-dimensional basement membrane cultures**

Three-dimensional basement membrane cultures of cell line models were established following the protocol described by Debnath et al (2003) with slight modifications. Wells in 24-well low attachment plates (Corning#3473) were pre-coated with a layer of 100% extracellular matrix (ECM) Matrigel (200 µl/well) (BD Biosciences #354230) and allowed to gelify at 37°C. Cells were trypsinized and diluted at densities of 1 x 10^5 or 2 X 10^5 cells ml^-1 in assay medium (DMEM/F12 supplemented with 5% HS, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin, 4 ng/ml EGF and 5% ECM-Matrigel). To ensure complete exposure of the cells to the inhibitors, one milliliter of the assay medium containing cells were mixed with Tocilizumab (final concnet. 50, 100 or 200 µg/ml),
Ruxolitinib (final concent. 0.5, 1 or 10 µM) or Tasquinimod (final concent. 0.5, 1 or 10 µM) and plated onto the pre-coated wells. Fresh assay medium containing the drugs at indicated concentrations was added every day. Pictures were taken after 6 days of treatment.

For each treatment we quantify the area of 50 acini using ImageJ software. A Gaussian distribution curve was generated based on the acini measurements and statistical differences were determined by a t-test.

**Mice**

Animal maintenance and experiments were performed in accordance with the animal care guidelines and protocols approved by Columbia University animal care unit. Eight-weeks old female NOD.CB17-Prkdcscid mice (Harlan) were injected with 5x10^6 cells, resuspended in Matrigel (BD Biosciences) : normal growth media (1:1) into the fat pad mammary gland. Doxycyclin was added to drinking water at a final concentration of 2.0 mg/mL. Tumor growth was monitored twice a week with callipers at the site of injection. Animals were sacrificed when tumor size reached 1.5 cm diameter.

**Antibody arrays**

MCF-10A and MCF-10A/ErbB2+ cells were cultured in the presence of DMEM:F12 media supplemented with 0.5% HS for 48 hours. Conditioned media was collected and hybridized on Human Cytokine P1 (42-Plex) Kit (Gentel Biosciences) following manufacturer instructions. Data was normalized to the background and the secretion ratio MCF-10A/ErbB2+ vs. MCF-10A was calculated.

**Chromatin Immunoprecipitation**

MCF-10A and MCF-10A/STAT3-V5 cells (untreated cells and cells treated with IL6 50 µg/mL for 30 minutes) were grown to 80-90% confluency in 150 mm culture dishes. After washing with PBS cells were fixed and crosslinked using trueChIP High Cell Chromatin Shearing Kit with SDS Shearing Buffer (Covaris #010128) following manufacturer instructions. Nuclei preparation and Lysis was performed with the same kit and an aliquot of sonicated extract was saved to be analysed (Input). In parallel we conjugated the anti-V5 antibody to the beads as follows: 5µL of protein G beads were incubated on ice for 3 minutes with 1 mL blocking buffer (0.5% BSA in 1x PBS) and collected using DynaMag
magnet for 30” twice. Finally beads were resuspended in 250uL of blocking buffer and mixed with 1 ug of anti-V5 antibody overnight at 4℃ on a rotator. The next day anti-V5 conjugated beads were collected and washed with blocking buffer three times as mentioned previously and finally resuspended in 1 mL of blocking buffer. 100µL of nuclear extracts were added to the bead solution and incubated overnight at 4℃ on a rotator.

After 12 hours beads were collected incubated in low salt wash buffer (20mM Tris-HCl pH8.0, 150mM NaCl, 2mM EDTA, 0.1% SDS and 1% triton-x100) for 15 minutes at 4℃ on a rotator. Subsequently beads were collected and consecutively washed-collected first with high salt buffer (20mM Tris-HCl pH8.0, 500mM NaCl, 2mM EDTA, 0.1% SDS and 1% triton-x100), with LiCl wash buffer (20mM Tris-HCl pH8.0, 250mM LiCl, 1mM EDTA, 1% Na-deoxycholate and 1% NP-40) and finally with TE buffer (10mM Tris-HCl pH8.0 and 1mM EDTA). Beads were centrifuged at 960 xg for 3 minutes and resuspended with 210 µL of elution buffer (50mM Tris-HCl pH8.0, 10mM EDTA and 1% SDS). The solution was incubated at 65℃ in a heat block for 15 minutes and vortexed every 2 minutes. Afterwards 180µL of elution buffer were added to WCE and we incubated input and pellet in the oven overnight at 65℃ after which lysates were centrifuged at top speed for 1 minute and the supernatants transferred into new tubes. Supernatants were digested and incubated at 37℃ for 2 hours in TE buffer containing RNase A (32mG/mL) and subsequently digested in proteinase K (200µG/mL) and incubated 2 hours at 55℃.

To proceed with DNA extraction we added 400uL of phenol/chloroform/isoamyl alcohol into a Phaselock tube (Prime #2302840). We transferred 400µL approximately of reverse crosslinked samples into the Phaselock tubes, mixed by hand shaking and spinned at top speed at RT for 10 minutes. We transferred the aqueous phase into a new tube and precipitated the DNA by adding 16uL of 5M NaCl, 1.5µL of 20uG/uL glycogen and 800µL of 100% EtOH. We incubated the samples at -20℃ for 30 minutes or overnight and then centrifuged the samples at top speed at 4℃ for 10 minutes. The pellets were washed with 500µL of 80% EtOH. Samples were spun again and the pellet resuspended in 40µL of TE buffer and incubated at 65℃ on a heating block for 15 minutes.

Primers used for Real-Time PCR:

S100A8 BS1 Fw: 5’- GATCAAGCAAGTGATGC -3’
Rv: 5' - TCACGTGCAATGAAAAT -3'
S100A8 BS2 Fw: 5' - CCTACCTGCTTTTCTTCT -3'
Rv: 5' - GCCAGAGTGTGCTACAGTCTC -3'
S100A9 BS1 Fw: 5' - GTTGCATAAATGAAAAGA -3'
Rv: 5' - ATCAGGACTTACCTTGAGGA -3'
S100A9 BS2 Fw: 5' - AACCAGTGCAAGTAGAAGAA -3'
Rv: 5' - GATTGATCCTTGTGAAGTC -3'
S100A9 BS3 Fw: 5' - AACCAGTGCAAGTAGAAGAA -3'
Rv: 5' - GATTGATCCTTGTGAAGTC -3'
S100A9 BS4 Fw: 5' - TTTGAGGATCTACACCAAT -3'
Rv: 5' - ATACCACTTCTGGCTCTCAA -3'
S100A9 BS5 Fw: 5' - TACTCCAGAAGCAAGTGT -3'
Rv: 5' - GGCTCAGTTCTAATGCACTC -3'
S100A9 BS6 Fw: 5' - GGAGAGTAGGGCTAGGAT -3'
Rv: 5' - GGAAGCTGGTTGTTTAGTTC -3'
S100A9 BS7 Fw: 5' - GAAGCTGGTTGTTTAGTTC -3'
Rv: 5' - AAGTCATCGTCTTGCACTC -3'
S100A9 BS8 Fw: 5' - ACTTCCCCCACTATTTCTGT -3'
Rv: 5' - ACTTCCCCCACTATTTCTGT -3'

Proliferation Assays
1 x 10^3 MCF-10A cells were seeded in 96-well black plates (Costar, 3603) in triplicate. Cells were incubated the CellTiter-Glo Substrate Reagent (CellTiter-Glo Cell Viability Kit, Promega, G7571), in the dark at 37°C for 10 minutes. Luminescence was read by luminometer (Modulus II Microplate Reader, Turner Biosystems).

Flow cytometry
To analyze apoptosis induced by Tocilizumab, Ruxolitinib and Tasquinimod 2 X 10^3 cells were plated in a 6 well plate and treated at the indicated doses for 15 days. Afterwards, floating and attached cells were stained with Annexin-V following the PE Annexin-V Apoptosis Detection Kit guidelines (BD-Pharmingen#559763).

**FIGURE SUPPLEMENTARY LEGENDS**

Figure Supplementary 1. Integrative functional studies to identify Master Regulators in HR-/HER2+ breast cancer cells. RNAi screens revealed that shRNAs targeting genes involved in (A) ERBB2 and (B) MAPK signaling pathways are preferentially depleted (positive hits highlighted in yellow) in MCF-10A/ErbB2* compared with parental MCF-10A. (C) Overlap of top differentially-expressed genes (left) or top predicted master regulators (right) for MCF-10A/ErbB2* versus wild-type MCF-10A cells in plastic plates (2D) and Matrigel (3D) cultures. (D) Enrichment plots of the three final candidates (STAT3, AGRN, GLRX) identified by RNAi screens and master regulator analysis in culture cells. All genes are ranked from the most up-regulated (left) to the most down-regulated (right) in MCF-10A/ErbB2* cells comparing to wild-type. Black bars on the bottom are ARACNe-predicted targets of each candidate from TCGA BRCA data. Enrichment is summarized by “P.GSEA”. “p” value and the blue dot vertical line (corresponding to z-score) indicates the expression of the candidate itself. “P.GSEA” is an integrated p-value of enrichment in 2D and 3D by Fisher’s method. (E) Schematic representation of competition assay where equal amount of parental cells (blue) and shRNA-transduced cells (red) are mixed and cultured together. The number of GFP+ (shRNA expressing cells) was measured by FACS and is represented in the line graph. Bar charts show the silencing efficiency of specific shRNAs as measured by qRT-PCR. *p<0.05. (F) Correlation of AGRN and GLRX activity (MR score) with the breast cancer subtype defined by HER2 and Hormone Receptors (HR) status in primary samples from METABRIC dataset. The number shown on the bars corresponds to the p-value for each correlation and the dashed line indicates the significance threshold.
Figure Supplementary 2. Hyperactivation of STAT3 is mediated by the oncogene HER2 in HR-/HER2+ cells. (A) Western blot analysis of STAT1, STAT3 and STAT5 phosphorylation and protein levels (left) in a series of MCF-10A isogenic variants as shown in the right panel. (B) Comparison of STAT3 phosphorylation levels in MCF-10A/ErbB2* and MDA-MB-231 cells. (C) STAT3 expression was tested after infection with inducible shRNA targeting STAT3 and addition of Dox for 5 days (50ng/ml). (D) Competition assay between parental MDA-MB-231 and variants where STAT3 expression has been reduced by the mean of shRNA. Red Fluorescent Protein signal is represented in the line graph.

Figure Supplementary 3. Autocrine secreted molecules activate STAT3 in MCF-10A cells transformed with HER2. (A) STAT3 phosphorylation in MCF-10A cells incubated for 1 hour with conditioned media of either parental MCF-10A or MCF-10A/ErbB2* cells growing for different times.

Figure Supplementary 4. Expression of S100A8 and A9 is modulated by STAT3. (A) Western blot of STAT3 phosphorylation when IL6 (50 µg/ml, 6 hours) is added to MCF-10A cells (left panel) or when its expression is inhibited by the mean of inducible shRNAs in MCF-10A/ErbB2* (right panel). (B) Quantification of mRNA levels by qRT-PCR in MCF-10A cells (blue bar) and MCF-10A/ErbB2* isogenic variant (red bar). (C) Correlation between S100A8 and S100A9 expression in primary tumors from breast cancer METABRIC dataset. (D) Schematic representation of SOCS3, S100A8 and S100A9 promoters cloned in pGL3 vector to control firefly luciferase expression. Blue squares indicate predicted STAT binding sites. (E) Western blot showing the phosphorylation of STAT3 in 293T cells after incubation with IL6 (50µ g/ml) for 6 hours. Cells were treated with Ruxolitinib (0.5 and 1 µ M) in the indicated lanes. (F) Determination of STAT3 phosphorylation in MCF-10A/STAT3-V5 cells after IL6 treatment for increasing times. Protein extracts were incubated with beads conjugatedor not to V5 antibody overnight and the immunoprecipitation product resolved in a gel.
Figure Supplementary 5. Generation of models to study S100A8 and A9. (A) The western blot shows the S100A8 and S100A9 levels in MCF-10A/ErbB2* cells infected with either the Dox inducible control vector (pTRIPZ) or the shRNAs against S100A8 and S100A9 (shS100A8/9). (B) Schematic representation of the generation and characterization of MCF-10A/ErbB2* cells expressing an shRNA against STAT3 where expression of S100A8/9 was restored by viral transduction of S100A8 and A9 cDNAs (see also supplementary methods). The bar chart represents the mRNA levels of STAT3, S100A8 and S100A9 in these cells analyzed by qRT-PCR. The genetic background of every variant is indicated below. (C) The panel shows the growth in soft agar of parental MCF-10A cells and a variant overexpressing S100A8/9 proteins. The western blot shows the expression level of both S100A proteins in the MCF-10A variant. MCF-10A/ErbB2* cells are used shown as positive control.

Figure Supplementary 6. Estimation of secreted levels of S100A8 and S100A9. (A) Estimation of S100A8 and S100A9 levels in MCF-10A and MCF-10A/ErbB2* conditioned media by Western blot. Different amounts of recombinant protein were loaded to generate a standard curve where sample protein quantity was extrapolated. Band intensity was determined by Quantity One software (BioRad). (B) Table describing Trastuzumab, Tocilizumab, Ruxolitinib and Tasquinimod commercial use and clinical indication. (C) Left pannel shows the quantification of Caspase3 positive cells after immunohistochemical analysis of MCF-10A/ErbB2* tumors after treatment with Tocilizumab, Ruxolitinib or Tasquinimod for 48h. Right pannel Quantification of Ki67 positive cells is represented in the right side. The bar chart represents me average +/-SD of 5 different samples. *p<0.05

Figure Supplementary 7. (A) Table indicating the molecular characteristics (ER and HER2 status) of the cell lines used in the study. (B) Western blot showing the effect that treating HR-/HER2+ HCC-1954 cells with Tocilizumab, Ruxolitinib or Tasquinimod has on the status of p-AKT and S100A8/9. Tocilizumab
(50µg/ml), Ruxolitinib (1 µM) or Tasquinimod (10 µM) were applied for 48 hours. (C) Complete set of images from eleven breast cancer cell lines plated into ECM-Matrigel and treated with Tocilizumab, Ruxolitinib or Tasquinimod for 6 days. Used doses are indicated on top of each picture. (D) Quantification of the effect of the different treatments in the growth of the acini shown in panel B is provided in the graphics showing the size distribution of 50 acini. *** p<10^{-5}; * p<0.05 (E) Box plot representing the final weight of tumors generated by MCF-10A/ErbB2* injected in the i.m.f.p. of SCID mice and treated with the indicated treatments. (F) HER2 levels of the Patient Derived Xenograft compared to frequently used breast cancer cell lines. Final weight of tumors generated by the breast cancer cell lines (G) or PDX (H) after their injection into SCID mice and treatment with Trastuzumab, Ruxolitinib or the combination of both. Pictures were taken at the moment of necropsy.
A) ERBB SIGNALING PATHWAY

B) MAPK SIGNALING PATHWAY
C

Top Diff-Exp genes (FDR<0.05)

2D
282

3D
458

18

3D
267

2D
246

Top Master Regulators (FDR<0.05)

P GSEA comb=0.003

AGRN GLRX

Number of genes: 10270 (total), 72 (gene set)

0.05
0.026
0.167
0.191
0.011
0.018
0.076
0.253
0.167
0.391
0.389
0.124
0.019

F AGRN GLRX

Days

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500
520
540
560
580
600
620
640
660
680
700
720
740
760
780
800
820
840
860
880
900
920
940
960
980
1000

E

Control cells
Infected cells

t0

Days

% of cells expressing GFP

0
10
20
30
40
50
60
70
80
90
100
110
120
130
140

0
1
2
3
4
5
6
7
8
9
10
11
12
13
14

F

AGRN

GLRX

HER2* vs HER2-
ER+ vs ER-
ER2+ vs ER2-
ER+ vs HER2-
ER- vs HER2-

-0.391
-0.263
0.078
0.389
0.124
### Table A

| Cell Line        | pERBB2 | pSTAT1 | pSTAT3 | pSTAT5 | STAT1 | STAT3 | STAT5 | β-Actin |
|------------------|--------|--------|--------|--------|-------|-------|-------|---------|
| MCF-10A          |        |        |        |        |       |       |       |         |
| MCF-10A/ErbB2*   |        |        |        |        |       |       |       |         |
| MCF-10A/RasV12   |        |        |        |        |       |       |       |         |
| MCF-10A/MycT58A  |        |        |        |        |       |       |       |         |
| MCF-10A/PI3K CA 20.1 |        |        |        |        |       |       |       |         |
| MCF-10A/ClnD1    |        |        |        |        |       |       |       |         |
| MCF-10A/E1A      |        |        |        |        |       |       |       |         |
| MCF-10A/PTEN null|        |        |        |        |       |       |       |         |
| MCF-10A/shp53    |        |        |        |        |       |       |       |         |
| MCF-10A/p53 DN   |        |        |        |        |       |       |       |         |

### Table B

| Cell Line        | ERBB2 | RAS | MYC | pAKT | CLND1 | E1A | p53 | β-Actin |
|------------------|-------|-----|-----|------|-------|-----|-----|---------|
| MCF-10A/ErbB2*   |       |     |     |      |       |     |     |         |
| MCF-10A/RasV12   |       |     |     |      |       |     |     |         |
| MCF-10A/MycT58A  |       |     |     |      |       |     |     |         |
| MCF-10A/PI3K CA 20.1 |     |     |     |      |       |     |     |         |
| MCF-10A/ClnD1    |       |     |     |      |       |     |     |         |
| MCF-10A/E1A      |       |     |     |      |       |     |     |         |
| MCF-10A/PTEN null|       |     |     |      |       |     |     |         |
| MCF-10A/shp53    |       |     |     |      |       |     |     |         |
| MCF-10A/p53 DN   |       |     |     |      |       |     |     |         |

### Table C

| Condition        | pERBB2 | STAT3 | β-Actin |
|------------------|--------|-------|---------|
| MDA-MB-231 + pTRIPZ |       |       |         |
| MDA-MB-231 + shSTAT3 #1 |     |     |         |
| MDA-MB-231 + shSTAT3 #2 |     |     |         |

### Table D

| Days | % of GFP cells |
|------|---------------|
| 0    | 0             |
| 5    | 10            |
| 10   | 30            |
| 15   | 50            |

---

**Legend**

- **Blue** line: MDA-MB-231 + pTRIPZ
- **Red** line: MDA-MB-231 + shSTAT3 #1
- **Green** line: MDA-MB-231 + shSTAT3 #2

**Notes**

- ERBB2, pSTAT3, and β-Actin levels are normalized to the respective control conditions.
- DOX treatment was applied as indicated (+) or not (-).
- The graph shows the % of GFP cells over the first 15 days of treatment.
| Time (h) | MCF-10A/ErbB2* Cond. Media | MCF-10A Cond. Media |
|---------|-----------------------------|---------------------|
| 0       |                             |                     |
| 12      |                             |                     |
| 24      |                             |                     |
| 48      |                             |                     |

**A**

- **pSTAT3** -
- **β-Actin** -
A

B

C

Rodriguez-Barrueco_Fig S5

2015/262642

DOX  -  +  -  +  -  +  -  +
S100A8-  
S100A9-  
β-Actin-

MCF-10A/ErbB2* shSTAT3 (DOX inducible)

cDNA-S100A8-GFP
cDNA-S100A9-dsRED

FACS

DOX
-  +  -  +  -  +  -  +
STAT3-
S100A8-
S100A9-
β-Actin-

C

MCF-10A MCF-10A/S100A8/9 MCF-10A/ErbB2*

S100A8-
S100A9-
β-Actin-

MCF-10A MCF-10A S100A8/9 MCF-10A ErbB2*
A

sS100A8 - sS100A9 -
10 ng 100 ng
75 ng
50 ng
25 ng
1x10^5 cells
MCF-10A
Cond. Media
ErbB2*
Cond. Media

B

| Compound   | Description                                                                 | Company          | Indication                                                                                           | Phase        |
|------------|-----------------------------------------------------------------------------|------------------|------------------------------------------------------------------------------------------------------|--------------|
| Trastuzumab| Humanized mAb that binds to ErbB2 extracellular portion                     | Genentech        | - HER2 overexpressing breast cancer
- HER2 overexpressing gastric cancer or GE adenocarcinoma                                                | Approved      |
| Tocilizumab| Humanized mAb that targets IL6-R                                            | Genentech        | - Rheumatoid arthritis, PJIA, SJIA
- Schizophrenia, graft vs host disease, Castleman disease, Erdheim-Chester disease, fibrous dysplasia of bone
- Ovarian cancer                                                                                          | Approved, Phase I-III |
| Ruxolitinib| Small molecule inhibitor of JAK1 and JAK2                                  | Incyte          | - Myelofibrosis
- Other tumors                                                                                             | Approved, Phase I/II |
| Tasquinimod| Small molecule inhibitor that binds and inhibits interactions of S100A8    | Ipsen, Active Biotech | - Prostate Cancer
- Hepatocellular, ovarian, renal cell, gastric cancer                                                      | Phase I-III, Phase II |

C

2015/262642

Rodriguez-Barrueco_Fig S6
| Cell line      | Molecular subtype | ER   | HER2 |
|---------------|-------------------|------|------|
| MCF-10A/ErbB2* | BaB               | -    | +    |
| MCF-10A       | BaB               | -    | -    |
| MDA-MB-361    | Lu                | +    | +    |
| BT-474        | Lu                | +    | -    |
| T47D          | Lu                | +    | -    |
| MCF-7         | Lu                | +    | -    |
| HCC-1954      | BaA               | -    | +    |
| SUM-190       | BaA               | -    | -    |
| Hs-578T       | BaA               | -    | -    |
| MDA-MB-231    | BaA               | -    | -    |
| HCC-70        | BaA               | -    | -    |

### Figure B

**HCC-1954**

| Condition   | Concentration |
|-------------|---------------|
| Untreated   | 0.5µM, 1µM    |
| Ruxolitinib | 1µM, 10µM     |
| Tasquinimod | 50µg/ml, 200µg/ml |
| Tocilizumab | 50µg/ml, 200µg/ml |

**SUM-190**

| Condition   | Concentration |
|-------------|---------------|
| Untreated   | 0.5µM, 1µM    |
| Ruxolitinib | 1µM, 10µM     |
| Tasquinimod | 50µg/ml, 200µg/ml |
| Tocilizumab | 50µg/ml, 200µg/ml |

### Figure C

**MCF-10A**

| Condition   | Concentration |
|-------------|---------------|
| Untreated   | 0.5µM, 1µM    |
| Ruxolitinib | 1µM, 10µM     |
| Tasquinimod | 50µg/ml, 200µg/ml |
| Tocilizumab | 50µg/ml, 200µg/ml |

**MCF-10A/ErbB2**

| Condition   | Concentration |
|-------------|---------------|
| Untreated   | 0.5µM, 1µM    |
| Ruxolitinib | 1µM, 10µM     |
| Tasquinimod | 50µg/ml, 200µg/ml |
| Tocilizumab | 50µg/ml, 200µg/ml |

### Figure D

**Graph D**

- **Density**
- **log2(Area)**

Legend:
- **Red** (Untreated)
- **Green** (Low dose)
- **Blue** (High dose)
