SORLA regulates endosomal trafficking and oncogenic fitness of HER2

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The human epidermal growth factor receptor 2 (HER2) is an oncogene targeted by several kinase inhibitors and therapeutic antibodies. While the endosomal trafficking of many other receptor tyrosine kinases is known to regulate their oncogenic signalling, the prevailing view on HER2 is that this receptor is predominantly retained on the cell surface. Here, we find that sortilin-related receptor 1 (SORLA; SORL1) co-precipitates with HER2 in cancer cells and regulates HER2 subcellular distribution by promoting recycling of the endosomal receptor back to the plasma membrane. SORLA protein levels in cancer cell lines and bladder cancers correlates with HER2 levels. Depletion of SORLA triggers HER2 targeting to late endosomal/lysosomal compartments and impairs HER2-driven signalling and in vivo tumour growth. SORLA silencing also disrupts normal lysosome function and sensitizes anti-HER2 therapy sensitive and resistant cancer cells to lysosome-targeting cationic amphiphilic drugs. These findings reveal potentially important SORLA-dependent endosomal trafficking-linked vulnerabilities in HER2-driven cancers.
he human epidermal growth factor receptor 2 (HER2; also known as ErbB2) is a receptor tyrosine kinase and a well-established oncogene. HER2 amplification is found in 15–20% of breast cancers12, and HER2 overexpression or activating mutations are clinically relevant in other solid tumours such as bladder cancer, gastric, colorectal, and lung adenocarcinoma3–5. The biological relevance of HER2 as a driver oncogene is undisputed, and several targeted therapies have been approved for treating HER2-dependent cancers.

Several previous studies, including ours, demonstrate that the oncogenic signalling and the endosomal traffic of many receptor tyrosine kinases are functionally coupled6–9. Endosomal trafficking critically controls, for instance, the strength and duration of signals emanating from the epidermal growth factor receptor (EGFR; also known as ErbB1) and MET6,10,11. However, in comparison to other receptor tyrosine kinases, the details of HER2 trafficking are poorly understood. The prevailing view, supported by two different models, is that HER2 resides almost exclusively in the plasma membrane in HER2-amplified cancer cell lines12. Data in favour of a ‘limited internalization’ model suggest that HER2 resists internalization due to (1) the absence of identified ligands required for induction of endocytosis13, (2) the absence of a recognizable internalization motif14, (3) inhibition of clathrin-coated pit formation15,16, (4) association with membrane protrusions17 and (5) heat shock protein 90 (HSP90)–dependent stabilization of HER2 on the plasma membrane18–20. On the other hand, data in support of a model for ‘rapid recycling’ resulting in nearly exclusive cell-surface localization of HER2 have been brought forward21–23. Hence, key outstanding questions are: does HER2 undergo endosomal trafficking in HER2-driven cancer cells, and what would be the functional consequence of HER2 trafficking for its oncogenic properties?

SORLA is a multifunctional intracellular sorting protein belonging to the sortilin and LDL-receptor families consisting of a large extracellular domain, a transmembrane domain and a short cytoplasmic tail24, which is essential for its membrane sorting functions25,26. SORLA has been implicated in regulating amyloidogenic functions25,26. SORLA has been implicated in regulating amyloid (AD) protein expression in cells with variable levels of SORLA expression (Fig. 1a, c; Supplementary Fig. 1d). In the BT474 and SKBR3 cells with high SORLA expression, HER2 was also distributed intracellularly in accordance with previous work17,21, and did not overlap with EEA1-positive and VPS35-positive intracellular vesicles (Fig. 1d). When SORLA-GFP expression was analysed in JIMT-1 cells (Supplementary Fig. 1f) showing Pearson’s R of 0.3 ± 0.01 indicative of partial co-localisation.

To study whether SORLA and HER2 would show similar dynamics in cells, we chose to image the MDA-MB-361 cells expressing intracellular as well as cell-surface pools of HER2. For visualization, we performed live-cell TIRF imaging (allowing visualization of events close to the plasma membrane) of SORLA-GFP and HER2 labelled with Alexa568-conjugated anti-HER2 antibody (trastuzumab; Tz-568). Short-lived SORLA- and HER2-positive structures were detected in the TIRF-plane, indicative of active dynamics to and from the plasma membrane. In addition, co-localizing puncta of SORLA and HER2 were frequently observed undergoing dynamic lateral movement on the plasma membrane (Supplementary Fig. 1g and Supplementary Movie 1). Live-cell imaging deeper in the cytoplasm showed that SORLA and HER2 move together within the same endosomal structures (Supplementary Fig. 1g and Supplementary Movie 2). Collectively, these data demonstrate that SORLA and HER2 undergo co-trafficking between the plasma membrane and endosomes.

### Results

**SORLA co-localizes with HER2 on the plasma membrane and in intracellular vesicles.** Analysis of different breast cancer cell lines revealed prevalent SORLA protein expression in cells with HER2-amplification (Fig. 1a). In addition, SORLA was highly expressed in the S637 bladder cancer cell line harbouring a HER2-activating mutation (S310F)24 when compared to the HER2-low T24 cell line and a primary patient-derived bladder cancer cell line (Supplementary Fig. 1a). As SORLA has not previously been scrutinized in carcinoma cells, we first examined cellular SORLA levels in a quantitative manner. Flow cytometry (FACS) analysis revealed BT474 cells to have the highest cell-surface levels of SORLA compared to MDA-MB-361 cells (intermediate expression) and JIMT-1 cells (lowest expression) (Fig. 1b), further validating the correlation between total SORLA and HER2 levels observed by western blotting (Fig. 1b; Supplementary Fig. 1b). Next, we investigated the subcellular localization of SORLA in different endosomal compartments. Endogenous SORLA was found to localize largely to early endosomes (identified by EEA1 and Rab5 expression) and retrograde vesicles (VPS35) in MDA-MB-361 breast cancer cells (Supplementary Fig. 1c). The SORLA-GFP fusion protein was similarly detected in EEA1- and VPS35-positive vesicles, and while present in recycling endosomes (Rab11) (Supplementary Fig. 1c), was not detected in late endosomes (Rab7) or lysosomes (LAMP1) (Supplementary Fig. 1c).

Previous studies have indicated that HER2 is mainly restricted to the plasma membrane12; however, these observations were based on a very limited number of cell lines and/or on exogenous HER2 overexpression13,17,21. Since SORLA, an important sorting protein in neuronal cells and adipocytes, is expressed in HER2-positive cancer cells (Fig. 1a), we were interested to re-examine the subcellular localization of endogenous HER2 in breast cancer cells with variable levels of SORLA expression (Fig. 1a, c; Supplementary Fig. 1d). In the BT474 and SKBR3 cells with high SORLA expression, HER2 was mainly on the plasma membrane, in accordance with previous work17,21, and did not overlap with EEA1-positive endosomes (Fig. 1a, c, Supplementary Fig. 1d). A similar distribution was apparent in the third SORLA-high cell line HCC1419 (Fig. 1a, Supplementary Fig. 1d). However, in cells with intermediate (MDA-MB-361) or low (HCC1954 and JIMT-1) SORLA expression, HER2 was also distributed intracellularly indicating that HER2 must be trafficked at least in a subset of HER2 cancers (Fig. 1c, Supplementary Fig. 1d). The distinct cell-surface HER2 levels were further validated with FACS (Supplementary Fig. 1e). With respect to the intracellular HER2 pool, a proportion of the receptor demonstrated a clear overlap with EEA1, indicating localization in early endosomes (Fig. 1c, d). In MDA-MB-361 cells, endogenous SORLA co-localized with HER2 in EAA1-positive early endosomes (Fig. 1d), and in the SORLA-low JIMT-1 cells, SORLA-GFP localized to HER2 and VPS35-positive intracellular vesicles (Fig. 1d). When SORLA-GFP expression was analysed in JIMT-1 cells (n = 130), 19.8 ± 0.8 percent of SORLA-GFP overlapped with intracellular HER2 (Supplementary Fig. 1f) showing Pearson’s R of 0.3 ± 0.01 indicative of partial co-localisation.

To study whether SORLA and HER2 would show similar dynamics in cells, we chose to image the MDA-MB-361 cells expressing intracellular as well as cell-surface pools of HER2. For visualization, we performed live-cell TIRF imaging (allowing visualization of events close to the plasma membrane) of SORLA-GFP and HER2 labelled with Alexa568-conjugated anti-HER2 antibody (trastuzumab; Tz-568). Short-lived SORLA- and HER2-positive structures were detected in the TIRF-plane, indicative of active dynamics to and from the plasma membrane. In addition, co-localizing puncta of SORLA and HER2 were frequently observed undergoing dynamic lateral movement on the plasma membrane (Supplementary Fig. 1g and Supplementary Movie 1). Live-cell imaging deeper in the cytoplasm showed that SORLA and HER2 move together within the same endosomal structures (Supplementary Fig. 1g and Supplementary Movie 2). Collectively, these data demonstrate that SORLA and HER2 undergo co-trafficking between the plasma membrane and endosomes.
The SORLA extracellular domain is required for SORLA–HER2 complex formation. Intrigued by the apparent co-trafficking of SORLA and HER2, we next performed a set of co-immunoprecipitation assays to investigate whether HER2 and SORLA associate. We found that endogenous HER2 and SORLA co-precipitate in MDA-MB-361 and BT474 cells, indicating that HER2 and SORLA may exist in the same protein complex (Fig. 1e). SORLA consists of an extracellular domain (ECD), a transmembrane domain (TM) and a short cytosolic domain (CD) (Fig. 1f). To dissect the SORLA–HER2 association further, we generated truncated SORLA-GFP fusions consisting of either the SORLA extracellular and transmembrane domains (ECD + TM) or the SORLA transmembrane and cytosolic domains (TM + CD) (Fig. 1f, g). HER2 co-precipitated with the full-length SORLA-GFP and with SORLA-GFP ECD + TM in cells, but failed to associate with SORLA-GFP TM + CD (Fig. 1g). Interestingly, SORLA-GFP TM + CD showed similar vesicular localization as full-length SORLA-GFP, whereas SORLA-GFP ECD + TM was found diffusely in membrane-compartment in the cytoplasm and on the plasma membrane (Supplementary Fig. 2a). Thus, while the SORLA ECD is necessary for the SORLA-HER2 protein complex, the SORLA CD appears to be required for correct subcellular localization of SORLA.

The SORLA ECD is subdivided into five domains: an N-terminal VPS10p domain followed by a β-propeller (BP), an EGF-like (EGF) domain, a complement type repeat-cluster (CR-C) and a FNIII-domain cluster (Supplementary Fig. 2b). To investigate which domain of SORLA is required for the SORLA–HER2 complex formation, we produced and purified myc and 6xHIS-tagged full-length SORLA ECD, and SORLA ECD fragments...
SORKA regulates HER2 cell-surface levels and HER2 oncogenic signalling. The apparent inverse correlation between SORKA levels and the proportion of intracellular HER2 in the different HER2 cell lines (Fig. 1a, c, Supplementary Fig. 1d) prompted us to hypothesize that cell-surface HER2 levels may be regulated by SORKA. To test this, we performed loss-of-function experiments in high-SORKA BT474 cells and gain-of-function experiments in intermediate/low SORKA cell lines MDA-MB-361 and JIMT-1 cells, respectively. In BT474 cells, with predominantly plasma membrane-localized HER2 and high SORKA expression, silencing of SORKA resulted in, approximately a 50% decrease in cell-surface HER2 protein levels (Fig. 2a). Conversely, in the SORKA-intermediate MDA-MB-361 and SORKA-low JIMT-1 cells, in which HER2 localizes more to endosomal structures, SORKA overexpression increased cell-surface HER2 levels significantly (Fig. 2a). Total HER2 protein levels followed a similar trend of being significantly downregulated in SORKA-silenced BT474 cells and upregulated in SORKA-overexpressing MDA-MB-361 and JIMT-1 cells (Fig. 2b, c). Although the reduction in total HER2 protein levels upon SORKA silencing was observed consistently, its extent varied among experiments. Quantitative PCR analysis of ERBB2 mRNA levels after SORKA silencing or overexpression did not show any significant differences indicating that SORKA-mediated regulation of HER2 occurs predominantly at the post-transcriptional level (Supplementary Fig. 3a). These effects of SORKA silencing may not be limited to regulation of HER2 alone; we find that cell-surface β1-integrin levels were also reduced upon SORKA silencing (Supplementary Fig. 3b).

HER2 amplification is a major driver of proliferation and tumorigenesis, which prompted us to explore whether SORKA plays a functional role in breast cancer cells. Efficient silencing of SORKA in HER2-amplified SORKA-high BT474 cells significantly reduced cell proliferation (Fig. 2d, Supplementary Fig. 3c –d). This was specifically due to loss of SORKA rather than off-target effects, given that silencing SORKA with five individual siRNAs significantly reduced BT474 cell proliferation (Supplementary Fig. 3d, e). Conversely, in the SORKA-low JIMT-1 cells, expression of SORKA-GFP, triggering HER2 upregulation on the plasma membrane (Fig. 2a), significantly increased proliferation of these cells (Fig. 2e, Supplementary Fig. 3f). When SORKA was silenced in non-HER2-amplified but FGFR2-amplified MFM-223 breast cancer cells (Fig. 1a), there was no effect on cell proliferation (Supplementary Fig. 3g), indicating that SORKA is required for proliferation only in HER2-dependent cancer cells. HER2 signalling on the plasma membrane along the PI3K/AKT pathway is critical for HER2 growth-promoting functions in cancer cells. Silencing of SORKA in BT474 and MDA-MB-361 cells led to decreased phosphorylation of AKT (Ser473) and 4E-BP1 (Thr37/46) as well as decreased cyclin D1 levels, but did not inhibit mitogen-activated protein kinase (ERK1/2) signalling (Fig. 2f, Supplementary Fig. 3h). Taken together these data suggest that SORKA silencing specifically attenuates cell proliferation and PI3K-dependent HER2 signalling in HER2-amplified cells.

To investigate further the requirement of SORKA expression for the proliferation of HER2-dependent cancer cells, we silenced SORKA in the intermediate SORKA expressers, MDA-MB-361 cells, with a 3′UTR targeting siRNA and then re-expressed SORKA in the same cells. Re-expression of SORKA-GFP in SORKA-silenced MDA-MB-361 cells fully rescued cell proliferation back to control levels (Fig. 2g, Supplementary Fig. 3i). Importantly, only full-length SORKA-GFP, and not the SORKA fragments lacking either HER2 binding function (SORKA-GFP TM + CD) or correct subcellular localization (ECD + TM), was sufficient to rescue the effect of SORKA silencing on MDA-MB-361 cell proliferation (Fig. 2h). This suggests that the direct or indirect interaction of SORKA with HER2 and SORKA sorting functions are both necessary for SORKA-mediated proliferation of HER2-dependent cancer cells.

Importantly, silencing SORKA compromised the in vivo tumour engraftment of HER2-amplified breast cancer cells in an orthotopic model. SORKA-silenced and control MDA-MB-361 cells were generated by transducing two short hairpin RNAs (shRNAs) targeting SORKA (shSORKA #1 and shSORKA #4) and a non-targeting control (shCTRL). Efficient SORKA silencing strongly inhibited in vitro proliferation of these cells (Supplementary Fig. 3j, k). When control-silenced cells were injected into the mammary ducts of immunocompromised NOD.SCID mice, multiple ductal carcinoma in situ (DCIS) lesions were formed within 10 weeks. In contrast, the development of DCIS tumours from SORKA-silenced xenografts was almost completely halted (Fig. 2i). Taken together these data indicate that SORKA functionally regulates both the expression and the oncogenic function of HER2 in breast cancer.

SORKA promotes HER2 recycling. The steady-state distribution of cell-surface receptors between endosomes and the plasma membrane is regulated by the respective rates of receptor internalization and recycling back to the plasma membrane. To study whether SORKA regulates this balance in HER2 cell lines, we investigated HER2 localization and trafficking in the presence and absence of SORKA. First, we silenced SORKA in BT474 cells with high SORKA levels and predominantly plasma membrane HER2 localization at steady state. Interestingly, shRNA-mediated SORKA silencing led to increased intracellular accumulation of HER2, normally not observed in these cells (Fig. 3a, b). A similar shift in HER2 subcellular localization was triggered when inhibiting vesicular recycling with primaquine, indicating that HER2 undergoes constant endocytosis balanced with very rapid recycling in cells with predominantly plasma membrane-localized HER2 and that SORKA may play a role in facilitating HER2 transport (Fig. 3c, d).

Next, we investigated HER2 dynamics in MDA-MB-361 cells, where HER2 is localized both endosomally and on the plasma membrane, and in which SORKA is expressed at intermediate levels. SORKA-silenced MDA-MB-361 cells were subjected to an imaging-based receptor uptake assay. Cell-surface HER2 was labelled with AlexaFluor 568-conjugated trastuzumab (Tz-568) on ice and receptor internalization was then induced by placing cells at +37°C for 15, 30, and 60 min before fixation. SORKA-silenced cells showed significantly greater accumulation of intracellular HER2 after 30 min of internalization when compared to control-silenced cells (Fig. 3e, f), suggesting a possible defect in recycling. To test this, we labelled cell-surface HER2 as above, allowed receptor trafficking for 45 min, removed the remaining cell-surface-bound Tz-568 antibody with an acid wash and then allowed receptor recycling to occur for 30 min. SORKA-silenced MDA-MB-361 cells demonstrated significantly greater retention of intracellular antibody, indicative of attenuated recycling (Fig. 3g). To investigate the role of SORKA in receptor traffic further, we utilized JIMT-1 cells, which express very low endogenous SORKA, contain a substantial fraction of intracellular
HER2 at steady state and where the overexpression of SORLA leads to increased HER2 cell-surface levels (Fig. 2a). Overexpression of SORLA-GFP in these cells prevented the intracellular accumulation of cell-surface biotinylated HER2 when compared to control transfected cells (Fig. 3h), suggesting possibly accelerated recycling. Furthermore, a biochemical recycling assay, in which biotin is cleaved off from an internalized biotinylated receptor upon recycling back to the plasma membrane, revealed that SORLA-GFP-expressing JIMT-1 cells recycle significantly more HER2 to the plasma membrane compared to JIMT-1 control cells (Fig. 3i). A more detailed time course (5, 10 and 15 min of recycling) indicated that HER2 recycling is higher in SORLA-GFP cells, compared to GFP cells, only at 10 min, possibly due to rapid re-endocytosis of the recycled HER2 in the SORLA-GFP cells (Supplementary Fig. 4a). Interestingly, SORLA silencing also attenuated the recycling of
β₁-integrins (without influencing endocytosis; Supplementary Fig. 4b, c), suggesting that in cancer cells SORLA could be linked to trafficking of other receptors in addition to HER2. Taken together, these findings demonstrate a role for SORLA in the regulation of HER2 recycling.

**Silencing SORLA triggers HER2 accumulation in dysfunctional lysosomes.** As SORLA silencing increased intracellular retention of HER2 in the internalization assays, we wanted to investigate the subcellular localization of HER2 in SORLA-silenced MDA-MB-361 cells. Imaging revealed striking accumulation of HER2 in enlarged LAMP-1-positive structures (late endosomes/lysosomes), not observed in control cells (Fig. 4a, Supplementary Fig. 5a). The accumulation of HER2 in these structures, within SORLA-silenced cells, is rather surprising as under normal conditions, lysosomal targeting of growth factor receptors is linked to rapid receptor degradation. However, HER2 protein levels in SORLA-silenced cells were only modestly reduced (Supplementary Fig. 5a), suggesting that attenuated HER2 signalling (Fig. 2f, Supplementary Fig. 3i) is not linked to receptor degradation. Instead, since the ligand for PI3K, PI(4,5)₂, is predominantly enriched on the plasma membrane and considered to be absent from late endosomes, this may explain the restricted HER2 signalling, through the PI3K/AKT pathway, from these compartments in SORLA-silenced cells.

Given the enlarged LAMP-1 structures and the apparent discrepancy between increased lysosomal localization of HER2 versus minimal effects on HER2 levels, we investigated whether loss of SORLA could be potentially linked to abnormal lysosome function. Our hypothesis was supported by strong perinuclear accumulation of LAMP1- and CD63 (LAMP3)-positive late endosomes/lysosomes in SORLA-silenced cells compared to control cells (Fig. 4b, c, Supplementary Fig. 5b, c). Lysosomal aggregation was confirmed with four different siRNAs targeting SORLA in both MDA-MB-361 and BT474 cells (Supplementary Fig. 5d). Thus, depletion of endogenous SORLA in breast cancer cells leads to altered subcellular localization of lysosomes. Interestingly, this is linked to the altered traffic of HER2, as dual silencing of HER2 and SORLA reduced lysosomal aggregation but did not fully revert the phenotype (Fig. 4d). This suggests that in SORLA-silenced cells, HER2 localization to lysosomes contributes to compromised lysosomal function. This is further supported by the fact that SORLA silencing in the non-HER2-amplified breast cancer cell line MFM-223 does not affect the subcellular localization of lysosomes (Supplementary Fig. 5e).

Further analyses using transmission electron microscopy (TEM) revealed enlarged lysosomes in SORLA-silenced cells suggesting potential lysosome maturation defects (Fig. 4e). To monitor the proteolytic activity of lysosomes, we analysed loss of quenching of a fluorogenic protease substrate, DQ Red BSA, loaded into cells. SORLA-silenced MDA-MB-361 cells showed significantly lower DQ Red BSA signal (indicating reduced lysosomal cleavage of BSA), detected either by confocal microscopy imaging (Supplementary Fig. 5f) or by flow cytometry, than the respective control cells (Fig. 4f). These data together indicate a link between SORLA-dependent HER2 signalling and lysosome integrity in HER2-driven cancer cells.

**Depletion of SORLA renders HER2-driven cancer cells sensitive to CADs.** Previous studies indicate that cancer cells possess functionally abnormal lysosomes making them more susceptible to cationic amphiphilic drugs (CADs), a heterogeneous class of molecules with a similar chemical structure resulting in lysosomal accumulation and increased lysosomal membrane permeabilization. Recently, cancer cells were shown to be more sensitive to CAD-induced cell death than non-transformed cells. Given the defective lysosomes of SORLA-depleted cells, we wanted to evaluate the response of anti-HER2 therapy-sensitive BT474 and therapy-resistant MDA-MB-361 cells to the antihistamine ebsaine, a CAD with cytotoxic effects in lung cancer. Interestingly, ebsaine displayed significantly lower IC₅₀ values when tested for growth inhibitory effects in both of these HER2-amplified breast cancer cells following SORLA silencing (Fig. 4g). Moreover, treatment of SORLA-silenced MDA-MB-361 cells with 15 µM ebsaine, which is close to the determined IC₅₀ value that inhibits the growth of these cells, significantly increased the levels of cleaved PARP₁, indicative of apoptosis, whereas no such effect was seen in control-silenced cells (Fig. 4h, i). Thus, depletion of SORLA increases the sensitivity of breast cancer cells to CADs, regardless of susceptibility to anti-HER2 therapy, presumably due to apoptosis triggered by lysosomal dysfunction. These data indicate that compromised lysosomal integrity downstream of SORLA depletion could be exploited therapeutically to induce cell death in anti-HER2 therapy-resistant breast cancer cells.

**High SORLA expression correlates with poor patient outcome specifically in HER2-amplified breast cancer patients.** Our in vitro data and observations of reduced tumour formation in mice demonstrate an important role for SORLA in regulating HER2 function in breast cancer cells. Next, we evaluated HER2 levels and SORLA expression in clinical specimens of breast cancer. Immunohistochemical staining of SORLA in a breast cancer tissue microarray (TMA) revealed that a substantial...
proportion (38%) of HER2-amplified breast cancers express moderate to high SORLA, indicating that HER2-amplified breast cancers fall into two subtypes with respect to SORLA positivity (Fig. 5a). However, in spite of a lack of overall correlation between HER2 amplification and SORLA expression in breast cancer, high SORLA expression appears to predict poor relapse-free and overall survival specifically within HER2-amplified breast cancer patients (Fig. 5b) according to the in silico biomarker assessment tool\(^{38}\) (http://kmplot.com), which interrogates large datasets (e.g. The Cancer Genome Atlas (TCGA), the European Genome Phenotype Archive (EGA) and the Gene Expression Omnibus (GEO)) in its analysis. Thus, SORLA levels could have a prognostic value in HER2-amplified breast cancers.

SORLA correlates with HER2 and regulates proliferation and tumour growth in bladder cancer. To broaden our study for other cancer types in which HER2 overexpression is common and
Fig. 3 SORLA promotes HER2 recycling. a, b Confocal microscopy images (a) and quantification (b) of HER2 staining after SORLA silencing (shSORLA #1 and shSORLA #4) in BT474 cells (n = 31 shCTRL, 25 shSORLA #1 and 20 shSORLA #4 cells from two experiments; analysis performed on 8-bit images; statistical analysis: Mann–Whitney test). c, d Confocal microscopy images (c) and quantification (d) of HER2 in vehicle- and primaquaine-treated (60 min) BT474 cells (n = 34 (vehicle), 28 (0.1 mM primaquaine) and 34 (0.3 mM primaquaine); analysis performed on 16-bit images; statistical analysis: Mann–Whitney test). e, f Microscopy analysis (e) and quantification (f) of AlexaFluor 568-labelled trastuzumab (Tz-568) internalization in MDA-MB-361 cells silenced with SORLA (siSORLA #3) or scramble (siCTRL) siRNA at the indicated time points (mean ± s.e.m; n = 64, 77, 86 and 64 siCTRL cells and 111, 83, 103 and 87 siSORLA #3 cells at the 0, 15, 30 and 60 min time points, respectively, from two independent experiments; statistical analysis: Mann–Whitney test; a.u. arbitrary units). g Microscopy-based HER2 recycling assay in control or SORLA siRNA-treated MDA-MB-361 cells. Labelled HER2 recycling back to the plasma membrane was monitored over a 30 min internalization step (45 min) and imaged with a confocal microscope. Ratio of surface/internalized Tz-568 signal is displayed as box plots (n = 34 and 57 siCTRL cells and 45 and 47 siSORLA cells for 0 and 45 min time points, respectively, from two independent experiments; statistical analysis: Nonparametric Kruskal–Wallis). h Immunoblotting analysis of biotin-labelled cell-surface HER2 internalization in JIMT-1 cells overexpressing SORLA-GFP (or control GFP-GFP-CTRL), and quantification of internalized HER2 relative to total HER2 (data are mean ± s.d.; n = 4 independent experiments; statistical analysis: Student’s t test). i Quantification of HER2 recycling rate (% return of internalized biotinylated cell-surface HER2 back to the plasma membrane after 10 min) in JIMT-1 cells transfected with GFP-CTRL or SORLA-GFP following 30 min of endocytosis (data are mean ± s.d.; n = 3 independent experiments; statistical analysis: Student’s t test). Scale bars: 10 µm. Box plots represent median and IQR and whiskers extend to maximum and minimum values. Where micrographs are shown, these are representative of n = 3 independent experiments; ROI magnified region of interest.

clinically relevant39,40, we stained SORLA in a bladder cancer TMA of 199 patients. In this cancer type HER2 and SORLA levels correlated significantly (chi-square test, p = 0.0092), whereas there was no correlation between SORLA and EGFR levels (Fig. 6a, Supplementary Fig. 6a). This led us to test if SORLA also plays a role in the regulation of HER2 cell-surface levels, cell proliferation and cell sensitivity to CADs or in vivo tumour growth in this cancer model. Silencing of SORLA in 5637 cells, a bladder carcinoma cell line with a HER2-activating mutation34, significantly inhibited their proliferation (Fig. 6b, c). In addition, cell-surface HER2 levels were consistently lower in SORLA-silenced 5637 cells than their control counterparts (Supplementary Fig. 6b), albeit these data did not reach statistical significance. Moreover, subcutaneous grafting of transiently SORLA-silenced 5637 cells (the strong anti-proliferative effect of shSORLA in vitro precluded sufficient propagation of stably silenced cells for in vivo experiments) in nude mice resulted in impaired tumour growth (mean tumour volume: control-silenced tumours, 78.7 mm³ and SORLA-silenced tumours, 47.7 mm³; p = 0.0461) (Fig. 6d, Supplementary Fig. 6c). Furthermore, SORLA-silenced 5637 tumours showed decreased proliferation, but not significantly increased apoptosis (Fig. 6e). Finally, in line with the breast cancer data, SORLA silencing sensitized 5637 cells to doxorubicin (Fig. 6f, g).

These results demonstrate that SORLA-mediated regulation of proliferation is not only restricted to HER2-amplified breast cancers, but is biologically important at least in HER2-driven urothelial cancer. It may be relevant to other neoplasms as well considering that data mining of the TCGA database revealed a significant positive correlation between ErbB2 and SORL1 expression in testicular germ cell tumours, cervical squamous cell carcinoma, endocervical adenocarcinoma, renal clear cell carcinoma, sarcoma and thymoma (Supplementary Fig. 6d).

Discussion

Here we demonstrate that SORLA, a sorting protein previously not investigated in carcinomas, is highly expressed in many HER2-driven cancer cell lines, and that SORLA regulates HER2 subcellular localization by forming a complex with the receptor and coupling it to the recycling machinery (Fig. 7). We find that HER2 distribution between the plasma membrane and endosomes is highly heterogeneous in cancer cells; those with lower SORLA expression exhibit a trend of harbouring a substantial pool of intracellular HER2 at steady state. Furthermore, silencing of SORLA induces intracellular accumulation of HER2, and overexpressing SORLA triggers cell-surface localization of HER2. Thus, HER2 undergoes rapid endosomal trafficking and recycling, the kinetics of which are regulated by SORLA-HER2 association. The fact that SORLA depletion (1) dramatically reduces proliferation of HER2-driven bladder and breast cancer cells in vitro and in vivo, (2) alters HER2 signalling, (3) interferes with lysosome integrity and (4) provides additive pro-apoptotic effects in combination with a clinically well-tolerated and widely used lysosome-accumulating drug provides new insight into the pathophysiology and targetability of HER2-driven oncogenesis.

Previous studies have shown discrepant results regarding HER2 trafficking. While some studies have shown that HER2 is resistant to internalization13,16,17, others have suggested rapid recycling of HER2 back to the plasma membrane22,23. In both scenarios, HER2 is mainly restricted to the plasma membrane where it can associate with signalling platforms to drive the proliferation and tumorigenesis of cancer cells. Here we investigated the localization of HER2 in six different HER2-amplified breast cancer cell lines and found very different patterns of localization. Our observation of HER2 overlapping with EEA1 and VPS35-positive endosomes in some of the cell lines suggests that at least a pool of HER2 moves back to the plasma membrane via retromer-dependent vesicle trafficking41. The heterogeneity in the subcellular localization of HER2 is interesting, since it might reflect the functions of HER2 and the efficiency of therapeutic targeting of HER2. Indeed, in our study, anti-HER2 therapy (lapanib, trastuzumab)-resistant JIMT-1, HCC1954 and MDA-MB-361 cell lines displayed more intracellular HER2 compared to the therapy-sensitive BT474, HCC1419 and SKBR3 cell lines with predominantly plasma membrane-localized HER2. This is also in line with two recent studies, published during the course of our investigation, that indicate a role for caveolin-1 in supporting HER2 internalization, and, in agreement with our findings, suggest that HER2 internalization and trafficking can be very different between different HER2-expressing cells42,43. Accordingly, the role of HER2 trafficking and the expression levels of specific trafficking proteins, such as caveolin-1 and SORLA, are important for the oncogenic activity of HER2 and most importantly for the response to anti-HER2 therapies. It is an intriguing possibility that the reduced viability of some HER2-dependent cancer cell lines upon depletion of trafficking proteins such as VPS35, Rab7 and LAMP144 might be related to alterations in HER2 trafficking.

The sorting functions of SORLA have been implicated in Alzheimer’s disease and obesity27–33. We found that the ECD of SORLA associates either directly or indirectly with HER2, while the intracellular domain of SORLA is required for the correct endosomal and plasma membrane localization of this molecule in
cancer cells. Importantly, re-expression of neither ECD-TM or TM-CD SORLA truncation mutants was sufficient to support proliferation of SORLA-silenced cells. This suggests that both the association of SORLA-ECD with HER2 and the correct subcellular localization of the SORLA-HER2 complex are necessary for proper SORLA function in supporting HER2-driven oncogenesis. Whether the observed coupling between SORLA-ECD and HER2 is mediated by direct binding or through other proteins remains to be investigated.

Lysosomal function is strongly linked to cellular fitness and it is especially important for the nutrient balance in rapidly growing cancer cells. One important finding of our work is that SORLA plays a major and unanticipated role in the maintenance of lysosome function in HER2-dependent cancer cells, but not in the FGF2-amplified, SORLA-positive MFM-223 cancer cells. The effects of SORLA depletion on lysosome function were two-fold. Lysosomes in SORLA-silenced cells showed perinuclear clustering. In addition, we found that lysosomes were enlarged, displayed an abnormal maturation defect-like appearance and had reduced proteolytic activity in SORLA-depleted cells. Strikingly, after SORLA silencing, HER2 accumulated into lysosomes without always being efficiently degraded (Fig. 7). The observed modest reduction in total HER2 protein levels was consistent, but varied in extent between experiments, which could be due to the parallel lysosomal defect. This is in stark contrast to the rapid lysosomal degradation of HER2 following treatment with geldanamycin, which inhibits HSP90-CDC37-complex-mediated stabilization of HER2 on the plasma membrane45,46 and suggests
Fig. 4 Silencing SORLA induces HER2 accumulation in dysfunctional lysosomes. a Immunofluorescence imaging of LAMP1 (white) and HER2 (red) in shCTRL and siSORLA MDA-MB-361 cells (n = 3 independent experiments). b Immunofluorescence imaging of LAMP1 (green) and CD63 (LAMP3; green) in MDA-MB-361 cells (blue is DAPI) after scramble (siCTRL) or SORLA (siSORLA #3 and siSORLA #4) siRNA silencing (n = 3 independent experiments). c Quantification of late endosomes/lysosome aggregation after SORLA silencing in MDA-MB-361 cells. LAMP1-positive structures ≥ 5 µm² were considered as lysosome aggregates (n = 73 siCTRL, 79 siSORLA #3 and 67 siSORLA #4 cells from three independent experiments; statistical analysis: Mann–Whitney test). d Immunofluorescence imaging and quantification of lysosomal aggregation in MDA-MB-361 cells treated with the indicated siRNA (n fields of view analysed, total cells = 11, 131 (siCTRL); 12, 182 (siSORLA #3); 11, 169 (siSORLA #3 + siHER2 #2); 10, 133 (siSORLA #4); 10, 121 (siSORLA#4 + siHER2 #2) from two independent experiments; statistical analysis: Mann–Whitney test). e Transmission electron microscopy imaging of lysosomes in siCTRL or siSORLA MDA-MB-361 and BT474 cells. Red arrows indicate the maturation defect in late endosome/lysosome structures (mean ± s.d.; n = 5 technical replicates). f Flow cytometry analysis of the fluorescence signal in DQ Red BSA-loaded (24 h) MDA-MB-361 cells after scramble (siCTRL) or SORLA (siSORLA #3 and siSORLA #4) silencing. Cells loaded with DQ Red BSA (4 h) and treated with bafilomycin (or vehicle) are included as controls (bafilomycin blocks lysosome function) (mean ± s.d.; n = 5 independent experiments; statistical analysis: unpaired Student’s t test). g Cell viability assay to determine ebastine (48 h treatment) IC50 values in SORLA- or control-silenced BT474 and MDA-MB-361 cells (mean ± s.d.; n = 12, four technical replicates, three independent experiments; statistical analysis: unpaired Student’s t test). h I Immunoblotting (h) and quantification (i) of cleaved PARP1 in ebastine-treated (15 µM, 48 h) siCTRL and siSORLA MDA-MB-361 and BT474 cells. α-tubulin is a loading control (mean ± s.d.; n = 3 independent experiments; statistical analysis: unpaired Student’s t test). Scale bars: 10 µm (a, b, d) and 1 µm (e). Box plots represent median and IQR and whiskers extend to maximum and minimum values. Nu nucleus.

Fig. 5 SORLA has a prognostic value in HER2-amplified breast cancer. a Immunohistochemical staining of SORLA and HER2 from a breast cancer tissue microarray (TMA; 883 patients in total). HER2-amplified tumours (199 patients) were categorized into negative/low (Neg/Lo) (staining intensity 0–1) and moderate/high (Mod/Hi) (staining intensity 2–3) groups. Numbers indicate staining intensity, 0 = negative, 1 = weak, 2 = moderate, 3 = high. b In silico biomarker assessment tool ((http://kmplot.com); including all datasets from 2010, 2012, 2014, 2017) analysis showing Kaplan–Meier plots of overall survival (OS; 10 years) and relapse-free survival (RFS; 20 years) of SORLA-high and SORLA-low patients (split by the best median cutoff) within all breast cancers (RFS n = 3953; OS n = 1402) and within HER2-amplified breast cancers (RFS n = 252; OS n = 129). Scale bars: 50 µm.

that SORLA regulates HER2 in a fundamentally distinct way than HSP90. Possibly due to mistargeted localization and the lack of suitable signalling co-factors on late endosomes, HER2 signalling to the PI3K/AKT/mTOR proliferative pathway was decreased in SORLA-silenced breast cancer cells. Interestingly, double silencing of SORLA and HER2 led to a less enlarged and clustered lysosomal phenotype suggesting that lysosomes might be overwhelmed in the presence of excess intracellular HER2. Whether the SORLA silencing alone induces a mild lysosomal defect leading to HER2 accumulation in lysosomes and mislocalised HER2 further enhances lysosomal stress remains to be elucidated. The possible role of SORLA-dependent alterations in lipid and glucose metabolism with respect to lysosomal dysfunction also remains to be investigated. SORLA could additionally contribute to the oncogenic properties of cancer cells by regulating the trafficking of other cargo. For example, we find that cell-surface β1-integrin levels are reduced and integrin recycling is attenuated upon SORLA silencing.

We found that SORLA-silenced cells undergo apoptosis when exposed to low doses of the antihistamine ebastine, which belongs to a heterogeneous group of CADs with similar chemical features and with a tendency to accumulate strongly in leaky lysosomes that are common in transformed cells and with a tendency to accumulate strongly in leaky lysosomes that are common in transformed cells 36,37. CADs, including ebastine, have been utilized in several in vitro, preclinical and clinical trials to target the vulnerability of lysosomes in cancer. Importantly, we found that both anti-HER2
therapy-resistant and sensitive HER2-amplified breast cancer cells are susceptible to the combination of SORLA silencing and low doses of ebastine. Silencing SORLA alone mainly reduced proliferation, but when combined with ebastine triggered enhanced apoptosis. This additive effect could potentially be exploited by combining current anti-HER2 therapies with CADs, since breast cancer cells and patient-derived xenografts resistant to an anti-HER2 drug-antibody conjugate were recently shown to possess dysfunctional lysosomes.

In conclusion, we have discovered that SORLA regulates the subcellular localization of HER2 and that dynamic recycling is essential for the oncogenic fitness of HER2. The endosomal trafficking of HER2 could provide new rationale for designing targeted therapies and understanding the resistance mechanisms induced by the current HER2-targeting therapies.
pain relief. Mice were sacrificed 10 weeks after tumour inoculation, and abdominal mammary glands were dissected. Mammary gland whole mounts were prepared on object glasses and fixed in Carnoy’s medium (60% EtOH, 30% chloroform, 10% glacial acetic acid) overnight (o/n) at −4 °C. After dehydration in decreasing EtOH series and staining with carmine alum (0.2% carmine, 0.5% aluminium potassium sulphate dodecahydrate) o/n at room temperature (RT), samples were dehydrated with glacial acetic acid overnight (o/n) at 37 °C, 5% CO2 until 70%.

Fig. 7 Schematic illustrating the role of SORLA in the oncogenic fitness of HER2 in cancer cells. SORLA, through interactions at its extracellular domain, is in a complex with HER2 and co-traffics with HER2, facilitating HER2 recycling to the plasma membrane to support HER2 downstream signalling. In the absence of SORLA, HER2 becomes localized to enlarged, partially dysfunctional lysosomes resulting in defective HER2 signalling and increased sensitivity to cationic amphiphilic drugs (CADs) like ebastine. End endosome, Lys lysosome

Cell lines and cell culture. MDA-MB-361 cells (ATCC, HTB-27) were grown in Dulbecco’s modified essential medium (DMEM; Sigma-Aldrich, D5769) supplemented with 20% foetal bovine serum (FBS; Sigma-Aldrich, F7524), 1% vol/vol penicillin/streptomycin (Sigma-Aldrich, P0781-100ML) and L-glutamine. BT474 (ATCC, HTB-20) and 3673 (ATCC, HTB-9) cells were grown in RPMI-1640 (Sigma-Aldrich, R8586) supplemented with 10% FBS, 1% vol/vol penicillin/streptomycin and L-glutamine. JIMT-1 (DSMZ, ACC 589), HCC1954 (ATCC, CRL-2338), HOCI419 (ATCC, CRL-2326), MCF7 (ATCC, HTB-22), MDA-MB-231 (ATCC, HTB-26), MDA-MB-436 (ATCC, HTB-130) and MFM-223 (DSMZ, ACC-422) were grown in DMEM supplemented with 10% FBS, 1% penicillin/ streptomycin and L-glutamine. MCF10A (ATCC, CRL-10317) and MCF10A DCIS. com (provided by Prof. J.F. Marshall, Barts Cancer Institute, Queen Mary University of London, London, UK) were grown in DMEM/F12 (Invitrogen, #11330-032) supplemented with 5% horse serum (Invitrogen#16050-122), 20 ng/ml human epidermal growth factor (Sigma-Aldrich, E9644), 0.5 mg/ml hydrocortisone (H0888–1G; Sigma-Aldrich), 100 ng/ml insulin (Sigma-Aldrich, I9278-5ML) and 1% vol/vol penicillin/streptomycin. All cells were regularly tested for mycoplasma infection and were grown at 37 °C, 5% CO2 until 70–80% confluence before being detached and replated. The medium was changed every 3 days. T24 (ATCC, HTB-4) and SKBR3 (ATCC, HTB-30) cells were grown in McCoy’s 5a Medium Modified (Sigma-Aldrich, M8403-500ML) supplemented with 10% FBS, 1% penicillin/ streptomycin and L-glutamine, and the patient-derived bladder carcinoma line (provided by Dr. P. Taimen, Turku University Hospital, Turku, Finland) was grown in F-medium (3:1 (v/v) F-12 Nutrient Mixture (Ham): DMEM (Invitrogen) supplemented with 5% FCS, 0.25 µg/ml hydrocortisone, 5 mg/ml insulin, 10 mg/ml gentamicin, 250 µg/ml fungizone, 8.6 mg/ml cholaer toxin, 125 ng/ml EGF) collected from irradiated Swiss 3T3 J2 mouse fibroblast feeder cell cultures and supplemented with 10 µM ROCK inhibitor (Y-27632, Enzo Life Sciences, Lausen, Switzerland).

Sensitivity to CADs

Generation of lentiviral shRNA and SORLA-GFP particles. Lentiviral particles containing sequences encoding shRNA against SORLA and GFP or a control scramble shRNA sequence and GFP or particles encoding for SORLA-GFP or GFP alone were generated in the 293FT packaging cell line (complete medium: high glucose DMEM, 10% FBS, 0.1 mM NEAA, 1 mM MEM Sodium Pyruvate, 6 mM l- glutamine, 1% penicillin/streptomycin and 0.5 mg/ml Geneticin) by transient transfection of transfer vector (#TL309181 SORLI S031650, #TL309181B SORLI S031650, #TL309181C SORLI S031650, #TL309181D SORLI S031650, or scramble control (#TR300201, Origene), second-generation packaging plasmidpsPAX2 (Addgene #12259) and envelope vector-pMD2 (Addgene #12260) with the ratio (7:2:1) using calcium-phosphate precipitation method. Seventy-two hours post transfection, medium containing viral vectors was collected, concentrated for 2 h by ultracentrifugation (26,000 × g) in a swing-out rotor SW-32Ti (Beckman Coulter, Brea, CA, USA), resuspended in residual medium and flash frozen in liquid nitrogen. Functional titre was evaluated in 293FT cells by FACS (BD LSRFortessa, Becton Dickinson).

Table 2. siRNA concentrations used ranged between 20 and 40 nM and cells were transfected with plasmids 24 h prior to experiments. Transient siRNA transfections were performed using Lipofectamine RNAiMAX reagent (Invitrogen, P/N 56532) according to the manufacturer’s instructions. SORLA-targeting siRNAs were ON-TARGETplus obtained from Dharmacon (#siSORLA #1 (J-004722-08), siSORLA #2 (J-004722-06), siSORLA #3 (J-004722-07), siSORLA #4 (J-004722-05). For rescue experiments, siRNA against the 3′UTR end of SORLA was obtained from Qiagen (siSORLA 3′ UTR, S05039888).HER2-targeting siRNAs were ON-TARGETplus obtained from Dharmacon (siHER2 #2, J-003126-17, siHER2 #4, J-003126-20). For controls, Allstars negative control (Qiagen, Cat. No. 102781) was used. siRNA concentrations used ranged between 20 and 40 nM and cells were transfected with siRNAs 48 h prior to experiments.

The corresponding oligonucleotide sequences can be found in Supplementary Table 2.

Western blot analysis. Protein extracts were separated using SDS-PAGE under denaturing conditions (4–20% Mini-PROTEAN TGX Gels) and were transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked with

Antibodies. The antibodies used are described in Supplementary Table 1.

Lentiviral transduction to generate stable cell lines. To generate stable silenced cell lines 4 × 10^6 BT474 or MDA-MB-361 cells were seeded on 10 cm dishes and transduced 24 h later with MOI 47 of lentivirus in a low volume of full media. To obtain stable overexpression of SORLA-GFP or GFP, 8 × 10^4 JMT-1 or MDA-MB-361 cells were seeded in a 24-well plate and transduced 24 h later with MOI 60. Medium containing viral particles was removed 16 h later. Cells expressing GFP, indicative of lentiviral integration were collected by fluorescence-assisted cell sorting (BD FACsaria II cell sorter, Becton Dickinson, Franklin Lakes, NJ, USA) with a gating strategy to obtain medium expression.

Transient transfections. For transient protein expression, Lipofectamine3000 (Invitrogen, P/N 100022052) and P3000 enhancer reagent (Invitrogen, P/N 100022058) were used according to the manufacturer’s instructions. Cells were transfected with plasmids 24 h prior to experiments. Transient siRNA transfections were performed using Lipofectamine RNAiMAX reagent (Invitrogen, P/N 56532) according to the manufacturer’s instructions. SORLA-targeting siRNAs were ON-TARGETplus obtained from Dharmacon—siSORLA #1 (J-004722-08), siSORLA #2 (J-004722-06), siSORLA #3 (J-004722-07), siSORLA #4 (J-004722-05). For rescue experiments, siRNA against the 3′UTR end of SORLA was obtained from Qiagen (siSORLA 3′ UTR, S05039888).HER2-targeting siRNAs were ON-TARGETplus obtained from Dharmacon (#HER2 #2, J-003126-17, siHER2 #4, J-003126-20). For controls, Allstars negative control (Qiagen, Cat. No. 102781) was used. SiRNA concentrations used ranged between 20 and 40 nM and cells were transfected with siRNAs 48 h prior to experiments.

The corresponding oligonucleotide sequences can be found in Supplementary Table 2.

Western blot analysis. Protein extracts were separated using SDS-PAGE under denaturing conditions (4–20% Mini-PROTEAN TGX Gels) and were transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked with
indicated primary antibodies overnight at 4 °C. Primary antibody dilutions used ranged from 1:500 to 1:1000. After primary antibody incubation, membranes were washed three times with TBST and incubated with fluorophore-conjugated second- ary antibodies diluted (1:1000) in blocking buffer at RT for 1 h. Membranes were scanned using an infrared imaging system (Odyssey; LI-COR Biosciences). The following secondary antibodies were used: donkey anti-mouse IRDye 800CW (LI-COR, 926-32212), donkey anti-mouse IRDye 680RD (LI-COR, 926-68072), donkey anti-rabbit IRDye 800CW (LI-COR, 926-32213) and donkey anti-rabbit IRDye 680RD (LI-COR, 926-68073). All original western blots for the manuscript can be found in Supplementary Fig. 7.

Co-immunoprecipitations. Cells were lysed in IP-lysis buffer (0.5% Triton X-100, 10 mM PIPES, pH 6.8, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA and complete protease and phosphatase inhibitors (Mediq; Roche)), cleared by centrifugation (13,226 × g, 10 min, 4 °C), and incubated with GFP-trap beads to pull-down GFP-proteins (Chromotek; gank 20) for 1 h at 4 °C or with mouse anti-HER2 antibody (1 µg/sample; Thermofisher Scientific, MA5-14057) or isotype matching IgG control antibody at +4 °C overnight. Antibody complexes were bound to 0.5% BSA pre-blocked protein-G sepharose beads for 1 h at 4 °C. Complexes bound to the beads were isolated using 1000 × g 3 min centrifugation, washed three times with washing buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % NP-40; 500 µl) and eluted in sample buffer. Input and precipitate samples were analysed by western blotting. Primary antibodies were incubated overnight at +4 °C. Mouse anti-HER2 (ThermoScientific, MA5-14057), rabbit anti-GFP (Molecular Probes; A11212) and mouse anti-SORLA (anti-LR1, BD Transduction Lab; 621633) diluted 1:1000 in 5% milk in TBST were used followed by the appropriate IRDye conjugated secondary antibodies.

Immunofluorescence staining and imaging. Cells were plated on µ-Slide 8-well (Ibidi, 80826) or in some cases in µ-dish 3.5 mm dishes (Ibidi, 80136). Cells were fixed with 4% paraformaldehyde (PFA) 10 min at RT, quenched with 50 mM NH₄Cl for 15 min at RT, blocked and permeabilized with 30% horse serum in PBS (Ibidi, 80826) or in some cases in µ-dish 3.5 mm dishes (Ibidi, 80136). Cells were incubated with primary antibodies (+0.3% Triton X-100 for 10 min at RT and incubated with primary antibodies diluted in 30% horse serum overnight at +4 °C. Staining was performed using antibodies against HER2 (trastuzumab, Roche, 0.15 µg/ml), mouse monoclonal antibody, Thermofisher Scientific, MA5-14057, 1:30 dilution), LAMPI (Santa Cruz; SC-20011 (H4A3), dilution 1:50), SORLA (rabbit monoclonal, CM Petersen Lab, Århus University, dilution 1:300), EEA-1 (goat polyclonal, Santa Cruz; sc-6415, dilution 1:30), VPS35 (goat polyclonal, Abcam; ab10099, dilution 1:300), CD63 (mouse mAb, Hybridoma Bank; HSC6, dilution 1:300) and Rab11 (rabbit polyclonal, Cell Signaling Technology, #5389, dilution 1:100). After several washes, appropriate secondary antibodies (donkey anti-mouse AlexaFluor 488 (Life Technologies, A21202), donkey anti-rabbit AlexaFluor 488 (Invitrogen), goat anti-human AlexaFluor 568 (Invitrogen, A21090), donkey anti-goat AlexaFluor 647 (Invitrogen, A21447), donkey anti-mouse AlexaFluor (Invitrogen, A31517)) diluted 1:300 in 30% horse serum were added together with DAPI (1:1000) for 1 h at RT. After PBS washes, samples were imaged right away or stored at −4 °C in the dark, stained either with a Carl Zeiss LSM780 laser scanning confocal microscope or a 3i CSU-W1 spinning disk confocal microscope with Hamamatsu CMOS (≥63x objective).

Transmission electron microscopy. Cells were fixed in 5% glutaraldehyde in 0.16 M m-collidine buffer, pH 7.4. The samples were post-fixed for 2 h with 1% OsO₄ containing 1.5% potassium ferrocyanide, dehydrated with a series of increasing alcohol concentrations and embedded in 45359 Fluka Epoxy Embedding Medium. Thin sections were cut with an ultramicrotome and stained with 1% uranyl acetate and 0.3% lead citrate. The sections were examined with a JEOL JEM-1400 electron microscope.

Live-cell imaging. Lentiviral transduced SORLA-GFP-expressing MDA-MB-361 cells were kept on ice and washed twice with ice-cold PBS. Alex 568-conjugated rhodamine (15 µg/ml) in Hank’s balanced salt solution (H9269; Sigma) for 30 min at 4 °C. After washing with cold PBS, cells were incubated with 0.15 µg/ml AlexaFluor 488 or AlexaFluor 568-conjugated trastuzumab (0.15 µg/ml) in Hank’s balanced salt solution (H9269; Sigma) for 1 h at 37 °C with 5% CO₂. The samples were washed four times with PBS and then fixed with 4% PFA (in PBS for 15 min at RT, washed and imaged in 1x Carl Zeiss LSM780 laser scanning confocal microscope).

Flow cytometry. For flow cytometry, cells were silenced and loaded with DQ Red BSA (25 mg/ml, 1 µl per well in six-well plates for 24 h) as above. When indicated, 25 mM bafilomycin (Calbiochem, 960000-10UG) or DMSO only was added to DQ Red BSA and the loading was shortened to 4 h. The loaded cells were detached with Trypsin, washed, fixed with 4% PFA for 15 min at RT, washed and analysed using LSFortessa (BD Biosciences). Following 200 laser scanning images, fluorescence signals from unstaed cells (background) were subtracted from the DQ Red BSA signals and geometric means were plotted.

Representative raw flow cytometry data can be found in Supplementary Fig. 8.

DNA extraction, cDNA synthesis and qPCR. Cells were lysed in RA lysis buffer and RNA was extracted according to the manufacturer’s instructions (Nucleospin RNA II kit, Macherey-Nagel, 740,955.5). RNA concentration was measured by NanoDrop Lite (Thermo). Complementary DNA (cDNA) was synthesized using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions. Quantitative real-time PCR reactions with TaqMan probes were performed according to the manufacturer’s instructions (Thermo/Applied Biosystems, TaqMan™ Universal Master Mix II, 444040). The following TaqMan probes (ThermoScientific, 4331182) were used: ErbB2 (Hs01001580_m1). Relative quantification of gene expression values were calculated using the ddCt method.51

Biotin-based HER2 endocytosis assay. HER2 endocytosis was measured using a cell-surface biotinylation-based assay as previously described. Briefly, JIMT-1 cells were transfected with GFP-CTRL or SORLA-GFP to grown to 80% confluence, placed on ice, and washed once with cold PBS. Cell-surface proteins were labelled with 0.5 mg/ml of EZ-link cleavable sulfo-NHS-s-s-biotin (#21331; Thermo Scientific) in Hanks’ balanced salt solution (H9269, Sigma) for 30 min at 4 °C. Unbound biotin was removed by washing with cold Hanks’ balanced salt solution. Thereafter, the cells were allowed to internalize receptors in pre-warmed 10% serum-containing medium at +37 °C for the indicated times. Internalization was stopped by transferring the cells to ice and adding cold medium. The remaining biotin on the cell surface was removed with 60 mM MesNa (63705; sodium 2-mercaptoethanol sulfonate: Fluka) in MesNa buffer (50 mM Tris-HCl [pH 8.6], 100 mM NaCl) for 30 min at 4 °C, followed by washing with 100 mM iododeoxacetic acid (IAA, Sigma) for 15 min on ice. To detect the total surface biotinylation, plates were left on ice after the biotin labelling and MesNa treatment was omitted. Cells were then washed with PBS, scraped in lysis buffer (50 mM Tris pH 7.5, 1.5 % Triton X-100, 100 mM NaCl, complete protease and phosphatase inhibitors (Mediq; Roche)) at 4 °C for 20 min. After clarification by centrifugation (14,000 g, 10 min, 4 °C), HER2 was immunoprecipitated from the supernatants with appropriate antibodies and protein G sepharose beads (17-0618-01; GE Healthcare). The immunoprecipitates were eluted in non-reducing Laemmli sample buffer and subjected to western blotting as described above. Biotinylated (internalized) HER2 and total receptor levels were detected in a 1:10 dilution with HRP-conjugated anti- biotin antibody (#7075; Cell Signaling Technology) and receptor-specific antibodies, respectively. Enhanced chemiluminescence-detected biotin and receptor signals were quantified as integrated densities of protein bands with ImageJ (v. 1.43 u) and each biotin signal was normalized to the corresponding receptor and total biotin signals.

HER2 recycling assay in JIMT-1 SORLA-GFP cells. HER2 recycling was measured using a cell-surface biotinylation-based assay as previously described. Cell-surface proteins were biotinylated and allowed to be internalized as described.
above for 30 min. After the first MesNa/IAA treatment, the internalized biotinylated fraction of receptors was then chased for the indicated time points by returning cells to pre-warmed media. 2 (moderate) or 3 (strong). SOLRA and HER2/EGFR staining was evaluated independently by two readers and the mean and maximum values of three tissue cores were determined for each patient. In the final statistical analysis, maximum scores for all the staining were used.

For the FinHer Breast Cancer tissue microarray immunohistochemistry, deparaffinized tissues were rehydrated with hydrogen peroxide to block endogenous peroxidase activity, and antigen was unmasked by using sodium citrate (10 mmol/L, pH 6.0) and 2100 Antigen Retriever instrument (Aptum Biologies Ltd., Southampton, UK). SOLRA antibody (rabbit polyclonal, Atlas Antibodies HPA 013321; 1:200 dilution) and Anti-EGFR (rabbit polyclonal, Ventana clone 4B5, 24 min incubation) were detected and visualized by using the BrightVision Poly-HRP anti-Rabbit kit (Immunologic) and 3,3′-diaminobenzidine (ImmPACT™ DAB, Vector Laboratories, Burlingame, CA, USA) following the manufacturer’s recommendations. The slides were counterstained with Mayer’s haematoxylin.

Tissue microarray construction. Bladder cancer TMA construction was approved by the Research Ethics Board of the Hospital District of Southwest Finland (1.8.2006/301). FFPE tissue samples from consecutive 199 patients who underwent radical cystectomy in Turku University Hospital between 1985 and 2005 were used and three 1 mm tissue cores per patient were punched for TMA. Average age at cystectomy was 64 and none received neoadjuvant therapies. FinHer series TMA has been described previously.

Quantification of intracellular HER2 levels. Several fields were randomly imaged with identical microscopic settings. ImageJ was used for analysis and quantifications. Intracellular Trz-568 fluorescence signal was analysed from maximal intensity projections of six planes taken from the middle of the cell (determined by DAPI signal) of intracellular endogenous fluorophore. Fluorescence intensity was measured from a single middle plane of the cell. Intracellular signal was quantified by manually gating the intracellular part of the cell and the intracellular signal was normalized to normalized to the cell area. Results are pooled from two independent biological replicates.

Quantification of late-endosome/lysosome aggregation. SOLRA silenced and control-silenced cells were fixed and stained for LAMP1 (Santa Cruz, SC-20011) and DAPI as described above. Cells were imaged with identical microscope settings with a Carl Zeiss LSM780 laser scanning confocal microscope. Image processing and quantifications were performed with the ImageJ software. The level of LAMP1-positive late-endosome/lysosome aggregation was quantified from the middle plane of the cell. To distinguish between individual late-endosomes/lysosome aggregates, watershed segmentation was used before quantification. The areas of late-endosome/lysosome aggregates were quantified from a single cell. A LAMP1-positive area larger than 5 µm² was considered as aggregate. Total area of LAMP1-positive late endosomes/lysosomes was calculated based on LAMP1 staining as well as the area of aggregates larger than 5 µm². The percentage of lysosomal aggregation was determined by the ratio of areas of aggregates larger than 5 µm² to total LAMP1-positive late-endosome/lysosome area.

Analysis of Ki-67 and TUNEL staining in 5637 cell xenografts. Pannoramic view (3DHISTECH Ltd) was used to scan histology slides and export images (×2 magnification) for image processing and quantifications, which were performed independently for all the samples with the ImageJ software. The TUNEL- and Ki-67-positive tumour areas were thresholded with MaxEntropy and quantified. The ratio of Ki-67-positive (or TUNEL-positive) area to total tumour area was calculated.

Co-localization analysis. Pixel-intensity-based Pearson correlation coefficient (R) between two channels was calculated using colo2 plugin (https://imagej.net/ Coloc_2) of ImageJ v1.51s with default parameters. Percentage co-localization between two vesicular particles was determined using ComDet (http://emtk.ekeptrika.com/ ekatrjuka/ComDet) plugin of ImageJ v1.51s. Particles were detected in both channels independently at approximated particle sizes of four pixels with sensitivities of signal/noise ratio of 4. Co-localization was determined based on a maximum distance between two particle centres of five pixels and expressed as a percentage.

Colonmy formation assay. Cells, silenced as indicated for 48 h, were plated on six-well plates (1 x 10⁴ cells/well) and exposed the following day to different concentrations of the indicated drugs and control cells were treated with DMSO. Drug-containing medium was replenished after 3–4 days and the cells were fixed and stained with 0.2% crystal violet in 10% EtOH for 10 min at RT after 7 days of treatment. Dried plates were then scanned, and the confluent areas of cells per well was quantified by using the Image (NIH) ColonyArea plug-in⁹⁹.

Statistical analysis. The GraphPad Prism software and two-tailed Student’s t test (paired or unpaired, as appropriate) was used for statistical analysis. Normal
distribution of the data was tested with the Shapiro–Wilk normality test. Unpaired t-test was used when normality could not be tested (n < 8). When data were not normally distributed, a Mann–Whitney test was used. In proliferation assays, two-way ANOVA was used. P values < 0.1 are shown in graphs.

**Data availability**

Breast cancer patient survival data are available from the Kaplan–Meier Plotter in silico biomarker assessment tool (http://kmplot.com) including all RNA expression datasets from 2010, 2012, 2014, and 2017. All the remaining data supporting the findings of this study are available within the paper and its supplementary information files, or from the corresponding author on reasonable request.

Received: 7 May 2018 Accepted: 23 April 2019
Published online: 28 May 2019

**References**

1. Joensuu, H. et al. Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. *N. Engl. J. Med.* 354, 809–820 (2006).
2. Slamon, D. J. et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 235, 177–182 (1987).
3. Bose, R. et al. Activating HER2 mutations in HER2 gene amplification negative breast cancer. *Cancer Discov.* 3, 224–237 (2013).
4. Lee, J. W. et al. Somatic mutations of ERBB2 kinase domain in gastric, adenocarcinomas. *Cancer Res.* 65, 1642–1646 (2005).
5. Haslekas, C. et al. The inhibitory effect of ErbB2 on epidermal growth factor receptor-ErbB2 oligomeric complexes at the plasma membrane. *Mol. Cancer Res.* 58, 622 (2009).
6. Pedersen, N. H. et al. Transformation-associated changes in sphingolipid metabolism sensitize cells to lysosomal cell death induced by inhibitors of acid sphingomyelinase. *Cancer Cell*. 24, 379–393 (2013).
7. Ellegraard, A. M. et al. Repurposing cationic amphiphilic antihistamines for cancer treatment. *EbioMedicine* 9, 130–139 (2016).
8. Gyory, B. et al. An online survival analysis tool to rapidly assess the effect of 22.777 genes on breast cancer prognosis using microarray data of 1,809 patients. *Breast Cancer Res. Treat.* 123, 725–731 (2010).
9. Yan, M. et al. HER2 expression status in diverse cancers: review of results from 37,992 patients. *Cancer Metastas.- Rev.* 34, 157–164 (2015).
10. Zhao, J. et al. Prognostic role of HER2 expression in bladder cancer: a systematic review and meta-analysis. *Int. Urol. Nephrol.* 47, 87–94 (2015).
11. Seaman, M. N. The retromer complex—endosomal protein recycling and beyond. *J. Cell. Sci.* 125, 4693–4702 (2012).
12. Sang, M. et al. Cavelo-mediates endocytosis as a novel mechanism of resistance to trastuzumab emtansine (T-DM1). *Mol. Cancer Ther.* 17, 243–253 (2018).
13. Pereira, P. R. M. et al. Cavelin-1 mediates cellular distribution of HER2 and affects trastuzumab binding and therapeutic efficacy. *Nat. Commun.* 9, 5137 (2018).
14. Marcotte, R. et al. Functional genomic landscape of human breast cancer drivers, vulnerabilities, and resistance. *Cell* 164, 293–309 (2016).
15. Cortese, K. et al. The HSP90 inhibitor geldanamycin perturbs endosomal structure and drives recycling ErbB2 and transferrin to modified MVBs/lysosomal compartments. *Mol. Biol. Cell* 24, 129–14 (2013).
16. Marx, C., Held, J. M., Gibson, B. W. & Renz, C. C. ErbB2 trafficking and degradation associated with K48 and K63 polyubiquitination. *Cancer Res.* 70, 3709–3717 (2010).
17. Funk, R. S. & Krize, I. P. Cationic amphiphilic drugs cause a marked expansion of apparent lysosomal volume: implications for an intracellular distribution-based drug interaction. *Mol. Pharm.* 9, 1384–1395 (2012).
18. Rios-Luci, C. et al. Resistance to the antibody-drug conjugate T-DM1 is based in a reduction in lysosomal proteolytic activity. *Cancer Res.* 77, 4639–4651 (2017).
19. Guzman, C., Bagga, M., Kaur, A., Westermarch, J. & Abanlka, D. ColonyArea: an Imagej plugin to automatically quantify colony formation in clonogenic assays. *PLoS ONE* 9, e92444 (2014).
20. Graham, F. L. & van der Eb, A. J. Transformation of rat cells by DNA of human adenovirus 5. *Virology* 54, 536–539 (1973).
21. Heid, C. A., Stevens, J., Livak, K. J. & Williams, P. M. Real time quantitative PCR. *Genome Res.* 6, 986–994 (1996).

NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-019-10275-0
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NATURE COMMUNICATIONS | (2019) 10:2340 | https://doi.org/10.1038/s41467-019-10275-0 | www.nature.com/naturecommunications
Arjonen, A., Alanko, J., Veltel, S. & Ivaska, J. Distinct recycling of active and inactive beta1 integrins. *Traffic** 13, 610–625 (2012).

Roberts, M., Barry, S., Woods, A., van der Sluijs, P. & Norman, J. PDGF-regulated rab4-dependent recycling of alphavbeta3 integrin from early endosomes is necessary for cell adhesion and spreading. *Curr. Biol.* **11**, 1392–1402 (2001).

**Acknowledgements**

We thank P. Laasola, J. Siivonen, and S. Collanus for excellent technical assistance, M. Saari for help with the microscopes. The Cell Imaging and Cytometry core facility, University of Turku, Turku Bioscience Centre for technical assistance with imaging. S. Hamalisto for useful suggestions regarding the CAD experiments. H. Hamidi for the scientific illustrations and manuscript editing, J. Westermarck, M. Salmi and the Ivaska lab for critical reading and feedback on the manuscript. This study has been supported by the Academy of Finland (M.P. and J.I.), Academy of Finland CoE for Translational Cancer Research (J.I. and H.J.), an ERC CoG grant 615258 (J.I.), the Sigrid Juselius Foundation, the Orion Research Foundation and the Finnish Cancer Organization (J.I.). P.S. has been supported by the Turku Doctoral Program of Molecular Medicine (TuDMM). E.P. is supported by the Finnish Cultural Foundation.

**Author contributions**

Conceptualization, M.P., J.I. and P.S.; Methodology, M.P., J.I., P.S., E.P., P.T., P.B., and O.M.A.; Investigation, M.P., J.I., P.S., E.P., I.P., N.Z.J., A.P., I.S., E.N., H.A.-A., J.I., M.G., H.S., H.J., and M.B.; Writing—original draft, M.P. and J.I.; Writing—review and editing, M.P., J.I., and P.S.; Resources, H.J., P.T., P.B., and J.I.; Funding acquisition, M.P. and J.I.; Supervision, M.P., J.I., and P.T.

**Additional information**

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-10275-0.

**Competing interests:** H.S. and H.J. own stocks of Sartar Therapeutics and are board members. H.J. has a co-appointment at Orion Pharma, and has received fees from Orion Pharma and Neutron Therapeutics Ltd. J.I., M.P., and P.S. have filed a patent application related to these findings. The remaining authors declare no competing interests.

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