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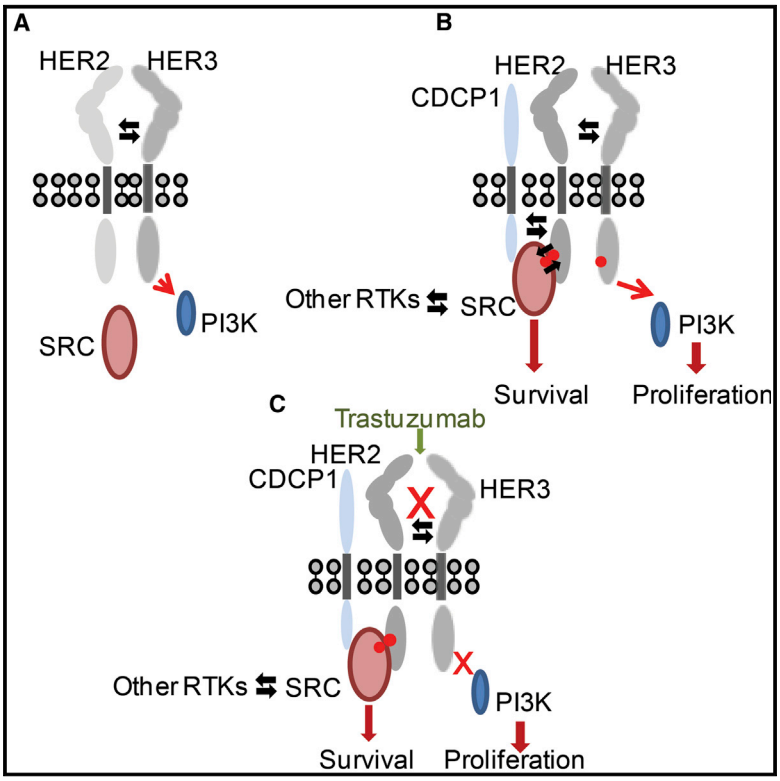
Interaction of CDCP1 with HER2 Enhances HER2-Driven Tumorigenesis and Promotes Trastuzumab Resistance in Breast Cancer

Graphical Abstract



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In Brief

Alajati et al. provide evidence that CDCP1 interacts with HER2 to increase HER2 signaling. CDCP1 overexpression confers trastuzumab resistance by promoting SRC-HER2 interaction. This study identifies CDCP1 as a biomarker for the stratification of HER2-positive breast cancer patients with poor prognosis and trastuzumab resistance.

Highlights

- CDCP1 and HER2 are co-overexpressed in primary and metastatic breast cancer patients
- CDCP1 overexpression enhances HER2 activity
- CDCP1 binds to HER2, promoting SRC-HER2 crosstalk
- CDCP1 overexpression confers resistance to trastuzumab

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Interaction of CDCP1 with HER2 Enhances HER2-Driven Tumorigenesis and Promotes Trastuzumab Resistance in Breast Cancer

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SUMMARY

Understanding the molecular pathways that contribute to the aggressive behavior of HER2-positive breast cancers may aid in the development of novel therapeutic interventions. Here, we show that CDCP1 and HER2 are frequently co-overexpressed in metastatic breast tumors and associated with poor patient prognosis. HER2 and CDCP1 co-overexpression leads to increased transformation ability, cell migration, and tumor formation in vivo, and enhanced HER2 activation and downstream signaling in different breast cancer cell lines. Mechanistically, we demonstrate that CDCP1 binds to HER2 through its intracellular domain, thereby increasing HER2 interaction with the non-receptor tyrosine kinase c-SRC (SRC), leading to trastuzumab resistance. Taken together, our findings establish that CDCP1 is a modulator of HER2 signaling and a biomarker for the stratification of breast cancer patients with poor prognosis. Our results also provide a rationale for therapeutic targeting of CDCP1 in HER2-positive breast cancer patients.

INTRODUCTION

Each year, breast cancer is diagnosed in over 1 million women worldwide. Although overall survival rates for breast cancer have improved significantly over the decades, more than 450,000 lives are still lost annually to this disease (Coughlin and Ekwueme, 2009). A better understanding of how breast cancer arises and progresses is needed to develop treatments for breast cancer patients. The epidermal growth factor receptor 2 gene (ERBB2 or HER2) is amplified and overexpressed in ~20% of invasive breast cancers, and is associated with metastasis and poor prognosis (Hynes and MacDonald, 2009). HER2 is a member of the ErbB receptor tyrosine kinase (RTK) family, which also includes EGFR, HER3, and HER4. Activation of HER2, induced by homo- or heterodimerization, drives transphosphorylation between pair receptor monomers, resulting in the recruitment to the receptor complex of signaling molecules such as PI3K, SHC, and GBR2 (Dankort et al., 1997; Segatto et al., 1993; Sepp-Lorenzino et al., 1996). Phosphorylated HER2 drives activation of the PI3K/AKT and MAPK/ERK pathways, thereby promoting cellular proliferation, survival, and differentiation (Hynes and Lane, 2005). Trastuzumab, an FDA-approved humanized antibody targeting HER2, is used in combination with chemotherapy to treat HER2-overexpressing breast cancers (Nahta and Esteva, 2006), with a high rate of response in early-stage breast cancer patients. However, a significant fraction of treated patients experience primary resistance to trastuzumab and ~70% of initial responders become resistant to the drug over time (secondary resistance) (Nahta and Esteva, 2006). Despite numerous research and clinical studies, additional attempts to interfere with the HER2 receptor alone have failed to yield a widely effective treatment. Therefore, the identification of targetable regulators of this pathway may reveal potential entry points for breast cancer therapy.

CUB domain-containing protein 1 (CDCP1) is a 140 kDa cell-surface glycoprotein with a large extracellular domain (ECD) containing two CUB domains, and an intracellular domain (ICD) containing five tyrosine phosphorylation sites. It has been demonstrated that tyrosine phosphorylation of CDCP1 is required for its full function and interaction with other proteins (Brown et al., 2004). Tyrosine 734 of CDCP1 (p-734) seems to be the main phosphorylation site for Src family kinases (SFKs). SRC was recently characterized as an essential regulator of CDCP1-driven tumorigenesis (Brown et al., 2004). A number of

studies showed that CDCP1 is overexpressed and tyrosine phosphorylated in multiple human cancers (Alajati et al., 2013; Rikova et al., 2007; Scherl-Mostageer et al., 2001), and that elevated CDCP1 expression in human tumors correlates with poor outcome and metastasis (Hooper et al., 2003; Miyazawa et al., 2010). While CDCP1 depletion reduces cancer cells dissemination in mice, CDCP1 overexpression increases the metastatic potential of different breast cancer cell lines in vivo (Deryugina et al., 2009; Uekita et al., 2008). Given the role of CDCP1 in cell migration and tumor growth, investigators have developed a number of compounds to target this protein in order to interfere with cancer progression, with promising outcomes (Kollmorgen et al., 2013; Siva et al., 2008). Recently, we reported that CDCP1 is highly phosphorylated in HER2-overexpressing breast cancer cells (Alajati et al., 2013). Moreover, CDCP1 was found to be upregulated and phosphorylated in a trastuzumab-resistant breast cancer cell line (Boyer et al., 2013), indicating a role of CDCP1 in the HER2 pathway. Here, we provide the first description of CDCP1 as a regulator of HER2 function. We found that overexpression of CDCP1 enhanced HER2 activity to promote cell migration in vitro and breast cancer progression in vivo. Consistently, CDCP1 depletion reduced HER2 activity and cancer progression in tumors driven by constitutive activation of HER2. In addition, we show that CDCP1 facilitates SRC-HER2 crosstalk, and CDCP1 overexpression confers resistance to trastuzumab treatment both in vitro and in vivo. This study provides further insights into the HER2 signaling pathway and a rationale to target CDCP1 in HER2-positive breast cancer patients.

RESULTS

CDCP1 Is Overexpressed in Primary and Metastatic Human Breast Tumors, and CDCP1/HER2 Expression Correlates with Poor Patient Prognosis

Recent studies have highlighted the role of CDCP1 in multiple human cancers (Wortmann et al., 2009). To investigate whether CDCP1 is overexpressed in human breast cancer, we first analyzed CDCP1 mRNA levels in three independent human breast cancer data sets (Finak, n = 59; TCGA, n = 450; and Curtis, n = 1,700) (Cancer Genome Atlas Network, 2012; Curtis et al., 2012; Finak et al., 2008), and found that in all three data sets CDCP1 was significantly overexpressed in human breast cancer compared with normal tissues (Figure 1A). To validate the protein level of CDCP1 in human breast cancer tissues, we first established CDCP1 immunohistochemistry (IHC) staining in a cell line expressing control or CDCP1 vectors (Figures S1A and S1B). Next, we determined CDCP1 protein levels by IHC in normal, primary, and metastatic human tissue microarrays (TMAs; n = 224). We classified CDCP1 IHC intensity staining as negative, +1, +2, or +3, and we considered positive only tumors having +2 and +3 positivity (CDCP1+) (Figure S1C). IHC analysis confirmed that CDCP1 protein levels were overexpressed in breast tumors when compared with normal tissues. Moreover, the percentage of cases that stained positive for CDCP1 was increased in metastatic tumor samples when compared with primary breast tumors and normal samples (Figure 1B). Interestingly, we found that in HER2-positive breast tumors (HER2+++), CDCP1 was overexpressed in 12% of primary and 30% of metastatic tumors, respectively (Figure 1C). Finally, we analyzed two large breast cancer gene expression data sets (Kao, n = 327; Curtis, n = 1483) (Curtis et al., 2012; Kao et al., 2011), including data from patients with a clinical follow-up of 15 and 25 years, respectively. Patients were classified into four groups depending on HER2 status (negative or positive) and CDCP1 expression levels (low or high). HER2-positive patients with CDCP1 overexpression had the lowest overall (Kao data set) and disease-free (Curtis data set) survival when compared with other groups (Figure 1D). Together, our data showed that HER2 and CDCP1 co-overexpression results in poor prognosis, suggesting that CDCP1 may cooperate with HER2 to promote tumor progression and metastasis.

CDCP1 Cooperates with HER2 to Drive Tumorigenesis

To assess the cooperativity between CDCP1 and HER2 in driving tumorigenesis, we established HEK293T cell lines stably overexpressing CDCP1 and HER2 alone or in combination (Figure 2A). Of note, only those cells expressing both HER2 and CDCP1 displayed an irregular/invasive-shaped morphology when grown in monolayer (Figure 2B). Furthermore, we performed a soft-agar assay using these cell lines. Whereas CDCP1 and HER2 alone displayed a similar increase in transformation ability, the combination of both resulted in a significantly higher number of colonies (Figure 2C). To validate these findings in a breast cancer cell line, we established MCF7 cells stably overexpressing control vector, CDCP1, HER2, or HER2/CDCP1 (Figure 2D). Co-overexpression of HER2/CDCP1 significantly increased colony formation (data not shown) and migration in vitro (Figure 2E). To assess the effect of HER2/CDCP1 co-overexpression in vivo, we injected these cells orthotopically into the mammary glands of immunodeficient mice. Although both CDCP1 and HER2 slightly increased tumor formation in vivo, overexpression of both strongly accelerated tumor onset and progression (Figure 2F). Taken together, these results show that CDCP1 cooperates with HER2 to enhance colony formation and cellular migration in vitro and tumorigenesis in vivo.

CDCP1 Downregulation Decreases HER2-Driven Cell Migration, Invasion, and Tumor Maintenance

To explore the effect of CDCP1 downregulation, we used the immortalized but nontransformed human breast epithelial cell line MCF10A, which expresses high levels of CDCP1, but not HER2. Next, we overexpressed two previously characterized HER2 active mutations (Greulich et al., 2012) in these cells to establish two cell lines: HER2-S310F (MCF10A-HER2-S310F) and HER2-S310Y (MCF10A-HER2-S310Y). Overexpression of both HER2 mutations increased the phosphorylation of HER2 at Y1248. This was mirrored by a concomitant phosphorylation of AKT (S473) (Figure 3A, left panel). We then infected these cell lines with doxycycline-inducible small hairpin RNA (shRNA)-CDCP1 lentivirus vector. CDCP1 knockdown was confirmed by immunoblot analysis and was associated with a strong decrease in the phosphorylation of HER2 (>3-fold compared with control) and p-AKT (S473) in both HER2-S310F and HER2-S310Y cells (Figure 3A, middle and right panels). These data were also validated in the HER2-positive MDA-MB361 cancer cells infected

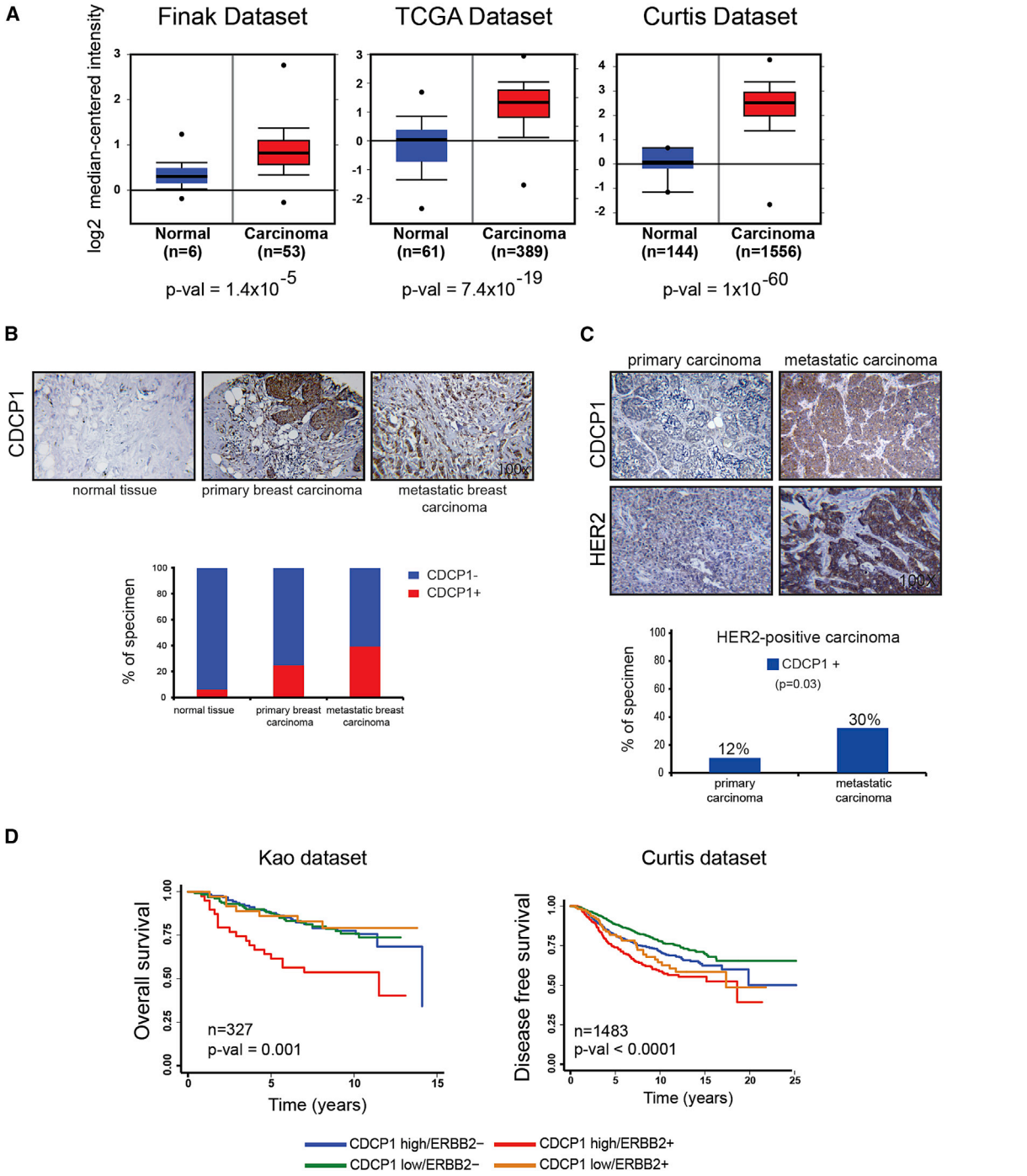

Figure 1. CDCP1 Is Overexpressed in Human Breast Cancer, and Patients with CDCP1/HER2 Co-overexpression Display a Worse Clinical Outcome

(A) CDCP1 is upregulated in human breast carcinoma. Box plots represent CDCP1 mRNA levels in normal and carcinoma samples from three independent data sets. The number of normal and carcinoma samples is indicated by n; p values were calculated using t test analysis.

(B) CDCP1 is upregulated in metastatic HER2-positive breast cancer. Upper panel: representative images of IHC staining for CDCP1 from normal tissue and primary or metastatic breast carcinoma. Lower panel: bar graph represents the percentage of CDCP1-positive specimens in a TMA (n = 224) including normal, primary, and metastatic breast cancer samples. Magnification is 100×.

(C) Upper panel: CDCP1 and HER2 staining correlates in breast tumors. Representative pictures of IHC staining for CDCP1 and HER2 in primary and metastatic breast carcinoma. Magnification is 100×. Lower panel: bar graph represents the percentage of CDCP1-positive specimens in primary and metastatic HER2-positive tumors. Fisher's test indicated a correlation between HER2 and CDCP1 staining in late-stage tumors (p = 0.03).

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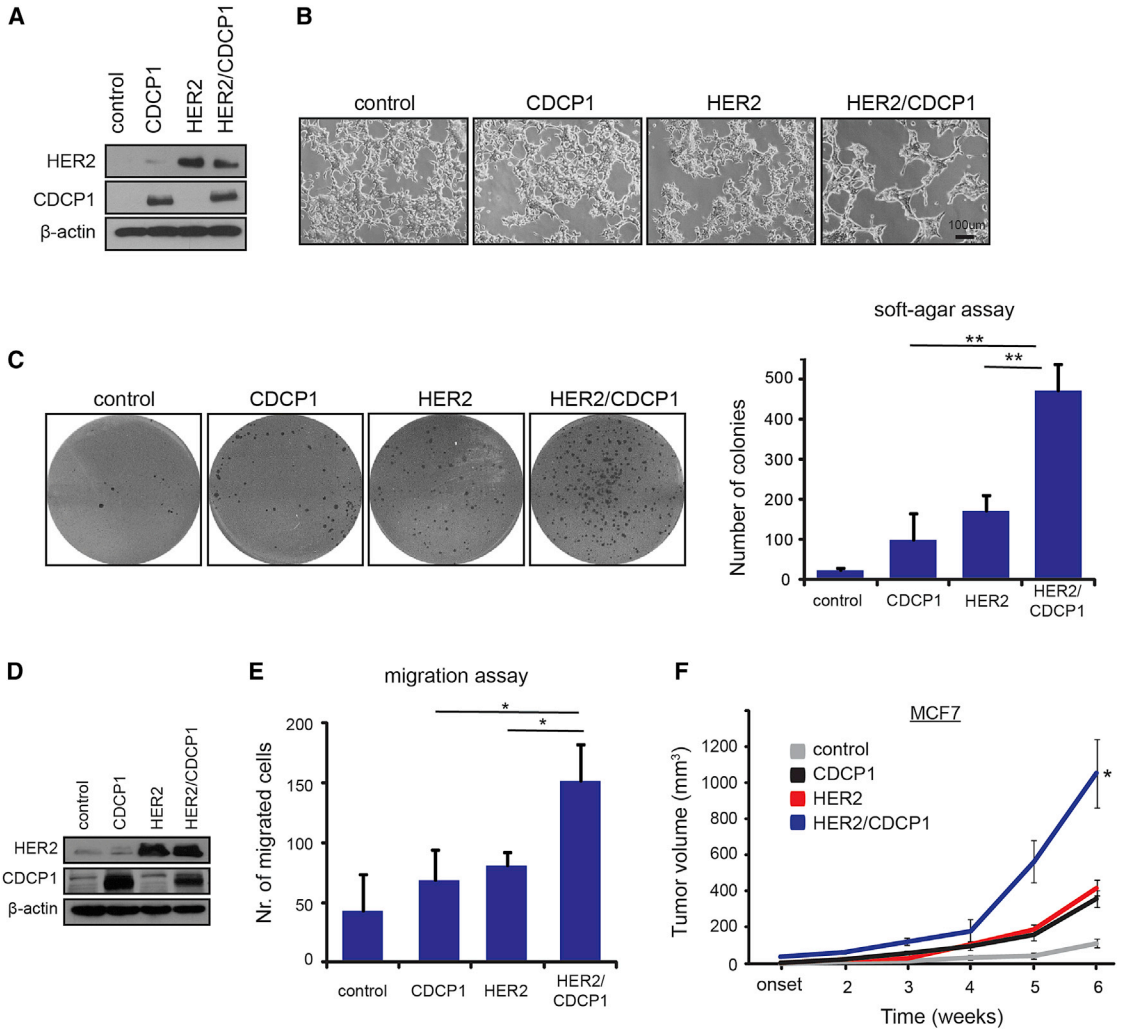

Figure 2. CDCP1 and HER2 Cooperate to Enhance Colony Formation In Vitro and Tumor Progression In Vivo

(A) Immunoblot analysis shows the establishment of HEK293T cell lines overexpressing CDCP1, HER2, or HER2/CDCP1.

(B) Representative phase-contrast images of HEK293T cell lines grown in monolayer. Scale bar, 100 μm.

(C) Left panel: representative images of HEK293T cell line colonies grown in soft agar for 30 days. Right panel: bar graph represents the number of HEK293T cell line colonies grown in soft agar for 30 days ± SEM (n = 5, **p < 0.01).

(D) Immunoblot analysis showing the overexpression of CDCP1, HER2, or HER2/CDCP1 in MCF7 cell lines.

(E) Transwell migration assays of MCF7 cells expressing control, CDCP1, HER2, or HER2/CDCP1. The bar graph shows the mean number of migrated cells ± SD (n = 3, *p < 0.05).

(F) Tumor growth curves (mm^3) of MCF7 cells expressing control, CDCP1, HER2, or HER2/CDCP1 vectors, showing mean tumor volumes (mm^3) ± SEM (n = 4, *p < 0.05).

with two shRNA-CDCP1s (Figures S2A and S2B). Interestingly, although the HER2-S310Y cells showed an increased motility (as compared with control cells), this effect was abrogated upon downregulation of CDCP1 in HER2-S310Y cells (Figure 3B). This result was further validated in a transwell-migration assay in which the HER2-S310Y cells lacking CDCP1 migrated less than control cells (Figure 3C).

MCF10A cells are an appropriate 3D in vitro model for studying breast cancer progression because they form well-organized acini that mimic the normal mammary structure in vivo (Debnath et al., 2002). On this line, overexpression of HER2-S310Y in MCF10A promoted the formation of irregular, invasive 3D acini structures (~80% of acinar structures; Figure 3D). However, CDCP1 knockdown strongly decreased the

(D) Patients with CDCP1 and HER2 co-overexpressing tumors display a worse clinical outcome. Patients were stratified depending on HER2 status and CDCP1 expression in two independent data sets. Kaplan-Meier curves show patients' overall (Kao data set) and disease-free (Curtis data set) survival. The number of patients in each cohort is indicated; p values were calculated using the log rank test.

See also Figure S1.

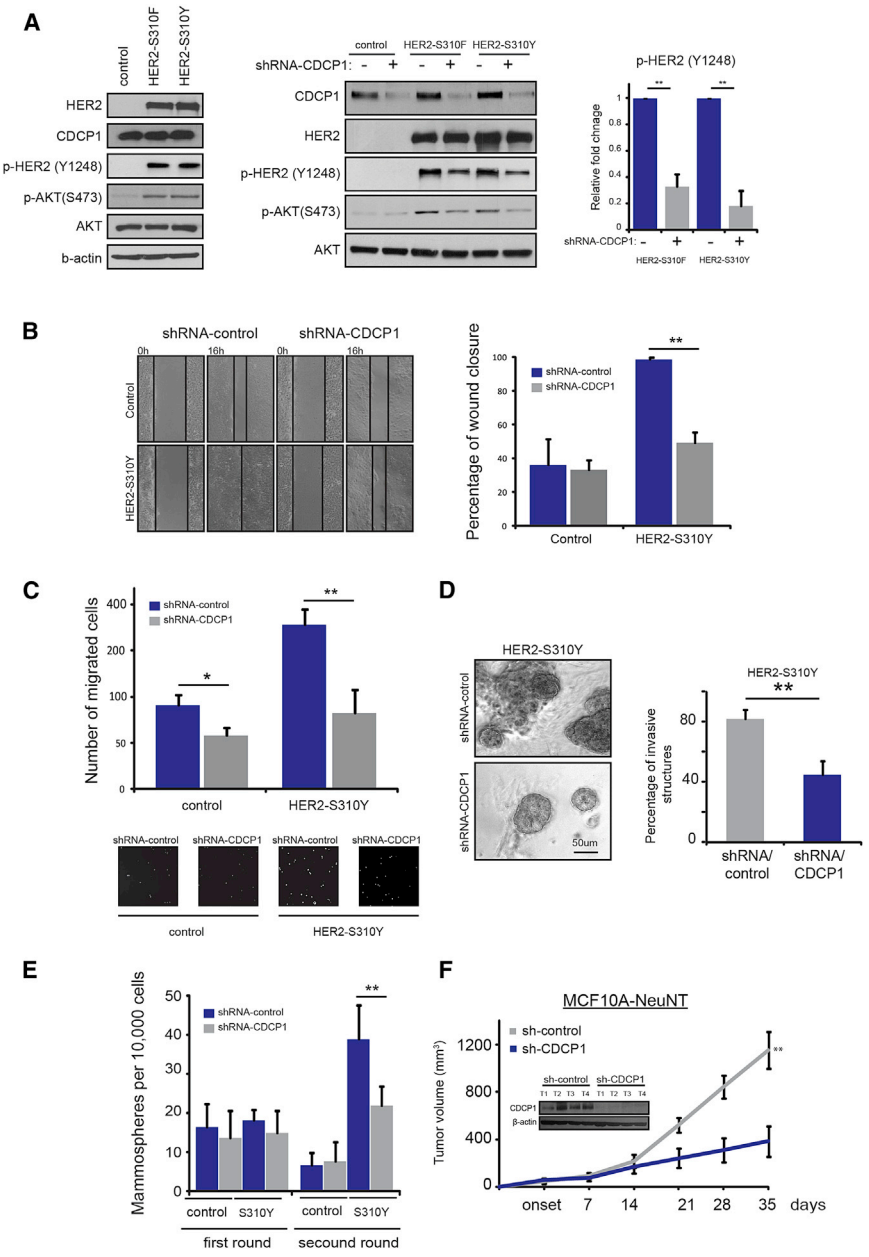

Figure 3. CDCP1 Downregulation Decreases Cell Migration, Invasion, and Self-Renewal Induced by HER2

(A) Left: immunoblot analysis of MCF10A cells overexpressing control vector, HER2-S310F, or HER2-S310FY mutations. Right: immunoblot analysis of MCF10A-control, MCF10A-HER2-S310F, or MCF10A-HER2-S310Y cells expressing doxycycline-inducible shRNA-control or shRNA-CDCP1. Bar graph represents the fold change of p-HER2 (Y1248) normalized to the total protein ± SD (n = 3, **p < 0.01).

(B) Wound-healing assay of MCF10A-control or MCF10A-HER2-S310FY cells expressing doxycycline-inducible shRNA-control or shRNA-CDCP1. Bar graph shows the mean percentage of wound closure ± SD (n = 3, ***p < 0.001).

(C) Upper panel: transwell migration assays of MCF10A-control or MCF10A-HER2-S310FY cells expressing doxycycline-inducible shRNA-control or shRNA-CDCP1. The bar graph shows the mean number of migrated cells ± SD (n = 3, *p < 0.05, **p < 0.01). Lower panel: representative images of DAPI-nuclei staining of migrated cells.

(D) Left: representative phase-contrast images of MCF10A-HER2-S310FY cells expressing doxycycline-inducible shRNA-control or shRNA-CDCP1 grown in 3D culture for 10 days. Right: the bar graph represents the percentage of invasive structures (n = 4, **p < 0.01). Scale bar, 50 μm.

(E) Mean of tumorsphere formation and the self-renewal capacity of MCF10A-control or MCF10A-HER2-S310Y cells expressing doxycycline-inducible shRNA-control or shRNA-CDCP1 (n = 3, **p < 0.01).

(F) Tumor growth (mm^3) in MCF10ANeuNT cells expressing doxycycline-inducible shRNA-control and shRNA-CDCP1. Inset: immunoblot analysis confirms the complete downregulation of CDCP1 in the tumor lysates.

HER2-S310Y-induced invasion (Figure 3D). It was previously reported that overexpression of HER2 active mutations in MCF10A cells increases the formation of tumorspheres (Aceto et al., 2012). In line with these findings, HER2-S310Y cells had an increased sphere-forming ability compared with the parental cells. However, CDCP1 downregulation strongly decreased the number of tumorspheres of HER2-S310Y cells (Figure 3E). Finally, to examine whether CDCP1 downregulation affects HER2-driven tumor progression and maintenance, we generated MCF10ANeuNT cells expressing inducible shRNA-CDCP1 or shRNA-control to downregulate CDCP1 after tumor establishment. These cells were injected orthotopically into the mammary fat pads of immunodeficient mice. After administration of doxycycline, tumor growth was not affected in tumors expressing shRNA-control, but it was markedly reduced in tumors expressing shRNA-CDCP1. Moreover, both tumor size (1,200 versus 400 mm^3) and weight (0.4 versus 0.19 g) were significantly reduced in shRNA-CDCP1 tumors compared with the control (Figure 3F). Taken together, these data demonstrate that CDCP1 downregulation directly affects migration, invasion, proliferation, and tumor progression in cells driven by active HER2 mutations.

CDCP1 Enhances HER2 Phosphorylation and Downstream Signaling, Promoting Tumor Formation and Progression In Vivo

To assess the effect of CDCP1 overexpression in HER2-positive breast cancer cell lines, BT474 and SKBr3 cells (*HER2* amplified breast cancer cells) were infected with retroviruses expressing either CDCP1-IRES-GFP (CDCP1) or IRES-GFP (used as a control) (Liu et al., 2011). Virus-mediated overexpression of CDCP1

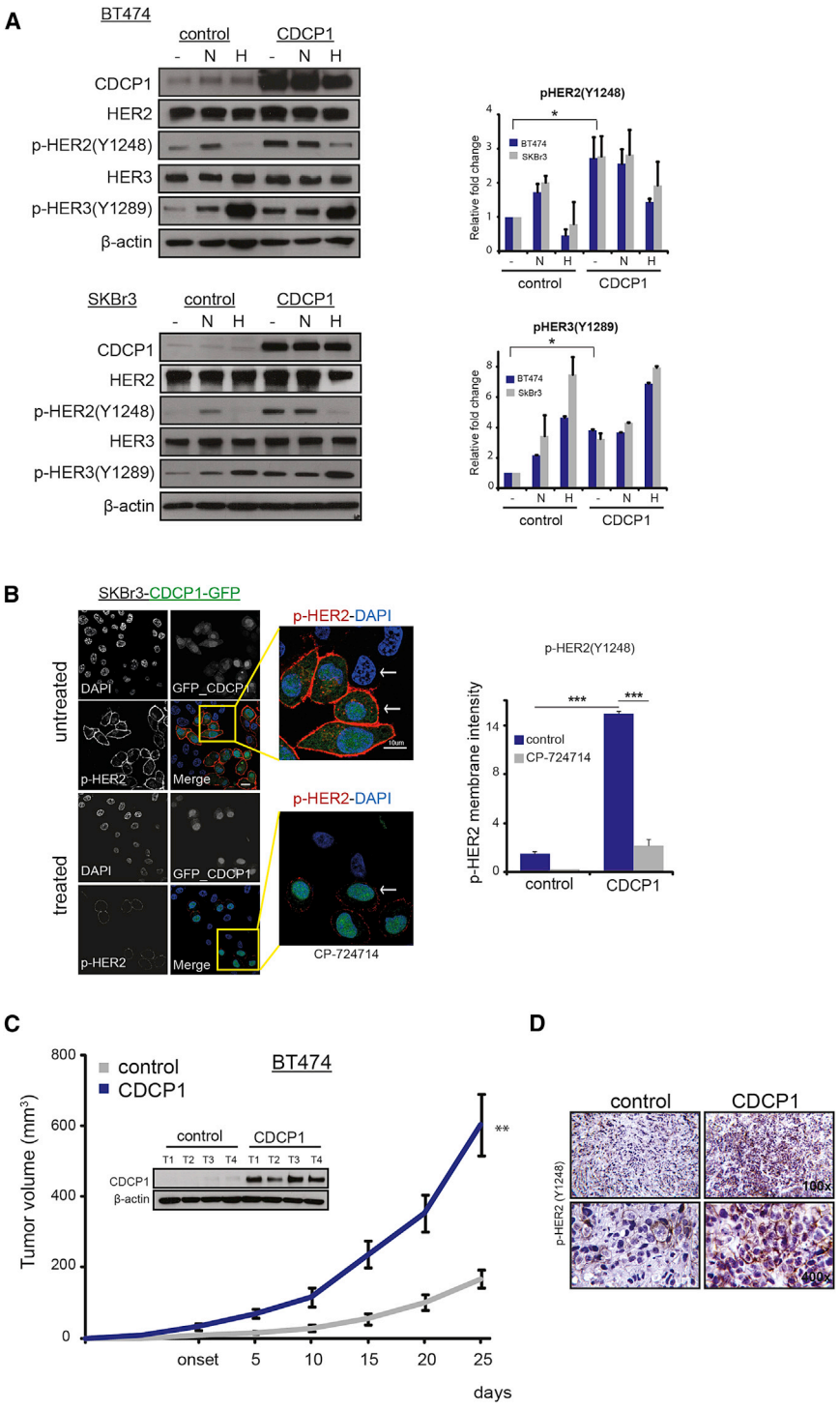

Figure 4. CDCP1 Enhances HER2 Activity in Breast Cancer Cell Lines and Affects HER2-Driven Tumorigenesis In Vivo

(A) Immunoblot analysis of BT474 and SKBr3 cell lysates overexpressing CDCP1 and control vectors. Cells were grown for 16 hr in serum-free medium (–), complete medium (N), or stimulated with heregulin (H, 10 ng/ml for 45 min). The bar graph shows the relative fold change of p-HER2 (Y1248) or p-HER3 (Y1289) normalized to total proteins ± SD (n = 3).

(B) Confocal images of immunofluorescence staining for p-HER2 (Y1248) in SKBr3 CDCP1-IRES-GFP cells grown in serum-starved conditions (16 hr) in the presence or absence of CP-724714 (1 hr treatment). The bar graph shows the quantification of p-HER2 (Y1248) membrane intensity staining of SKBr3-overexpressing IRES-GFP or CDCP1-IRES-GFP constructs starved for 16 hr in the presence or absence of CP-724714 (n = 3, ***p < 0.001). White arrows indicate both CDCP1-IRES-GFP-positive and -negative cells. Scale bar, 10 μm.

(C) Left: tumor growth curve (mm^3) of BT474-control and BT474-CDCP1. Inset: immunoblot analysis shows CDCP1 expression in the tumor lysates BT474-control and BT474-CDCP1 (n = 9, **p < 0.01).

(D) Representative IHC images of p-HER2-stained sections of BT474-control and BT474-CDCP1 tumors dissected 25 days after tumor onset. Magnification is 100×.

See also Figures S2–S5.

in both cell lines led to an increased phosphorylation of HER2 at Y1248, and HER3 at Y1289 compared with the control (Figure 4A). Notably, HER2 and HER3 phosphorylation was strongly enhanced (≥3-fold) in cells cultured under serum-starved conditions (Figure 4A). Consistently, the PI3K/AKT and MAPK/ERK pathways were enhanced in BT474-CDCP1 and SKBr3-CDCP1 cells (Figure S2C). These data were confirmed by an RTK signaling antibody array in SKBr3-CDCP1 cells, demonstrating an increase in the phosphorylation of HER2 pathway proteins such as HER2, HER3, EGFR, AKT, ERK1/2, and S6 compared with the control (Figure S2D). Next, we used immunofluorescence analysis to determine the effect of CDCP1 on HER2 activation. SKBr3 cells were infected with a low titer of CDCP1 vector to ensure the overexpression of CDCP1 in only a subpopulation of cells. First, we confirmed HER2 and CDCP1 expression by immunofluorescence in SKBr3-control and SKBr3-CDCP1 (Figure S3A). Then, we performed immunofluorescence analysis for p-HER2 and p-HER3 in both of these cell lines. Whereas control cells showed negative staining for p-HER2 and p-HER3, SKBr3-CDCP1 cells showed a marked increase in p-HER2 and p-HER3 staining (Figures 4B [upper panel], S3B, and S3C). Interestingly, in SKBr3-CDCP1 cells, p-HER2 and p-HER3 staining increased only in CDCP1-infected cells (GFP-positive cells; Figure 4B, upper panel). This effect was abrogated when cells were treated

with the HER2 kinase inhibitor CP-724714 (Figure 4B, lower panel). Together, these results showed a clear involvement of CDCP1 in the activation of HER2 signaling pathway. To validate these findings, we performed a gene expression profile analysis in SKBr3-CDCP1 and control cells. Interestingly, the gene expression profile of cells infected with CDCP1 was similar to the signature of HER2-positive breast cancers in a subset of patients (Figure S4A). Moreover, Gene Ontology enrichment (GOE) and gene set enrichment analysis (GSEA) showed that CDCP1 overexpression in SKBr3 significantly enhanced the expression of gene sets involved in DNA replication, cell cycle, proliferation, epithelial-to-mesenchymal transition (EMT), and metastasis, which are normally enriched in tumors driven by HER2 (Figure S4B). Finally, to assess whether CDCP1 overexpression in HER2-positive breast tumors enhances tumor progression in vivo, we orthotopically injected BT474-CDCP1 and control cells into the mammary glands of immunodeficient mice. Tumor growth was monitored for up to 25 days. Notably, CDCP1 overexpression significantly accelerated tumor onset and increased both tumor size and weight when compared with the control (Figures 4C and S5A). CDCP1 expression in tumors was confirmed by western blot analysis (Figure 4C, inset). Ki-67 IHC analysis revealed an increased proliferation of BT474-CDCP1 tumors compared with control (30% ± 10% versus 60% ± 6%; Figure S5B), whereas staining for cleaved caspase-3 showed no differences in apoptosis. Finally, IHC analysis for p-HER2 (Y1248) showed that CDCP1 overexpression also enhanced p-HER2 staining in vivo (Figure 4D). Taken together, these results demonstrate that CDCP1 enhances HER2 phosphorylation, thereby promoting tumor formation and progression in vivo.

CDCP1 Binds to HER2

Our results thus far have shown a crucial role of CDCP1 in HER2 phosphorylation and pathway activation. Therefore, we investigated whether CDCP1 could enhance the levels of known regulators of the HER2 signaling pathway (e.g., RTKs levels, Heregulin, and EGF). Gene expression analysis of possible activators of the HER2 pathway showed that none of the major drivers of HER2 activation were significantly up- or downregulated upon CDCP1 expression (Figure S5C).

We further sought to determine whether CDCP1 and HER2 could directly interact. To assess a possible physical interaction between CDCP1 and HER2, we first validated the localization of both proteins. Confocal microscopy analysis of immunofluorescence staining for both CDCP1 and HER2 confirmed their cell membrane co-localization in MDA-MB361 cells (Figure 5A, left panel). Further quantification showed a marked overlapping staining intensity of both proteins (Figure 5A, right panel). We then performed immunoprecipitation analysis in three different cell lines expressing both endogenous (MDA-MB361) and exogenous (SKBr3, BT474) protein levels of CDCP1. Immunoprecipitation of HER2 followed by immunoblotting of CDCP1 revealed a clear interaction of both proteins in MDA-MB361, BT474-CDCP1, and SKBr3-CDCP1 cell lines (Figures 5B and 5C). Interestingly, CDCP1 was also detectable in BT474-control cells after HER2 immunoprecipitation. We next assessed whether CDCP1 could affect HER2 dimerization, which would explain the increased HER2 phosphorylation observed in CDCP1-overexpressing cells. In line with this hypothesis, we found that CDCP1 overexpression enhanced HER2 dimerization (either homo or hetero) under native conditions as shown in BT474-CDCP1 cells (Figure S5D). Next, we assessed whether CDCP1 could enhance HER2/HER3 or EGFR/HER2 heterodimerization. To this end, we performed HER2 immunoprecipitation followed by immunoblotting for HER2, CDCP1, EGFR, and HER3 in BT474-CDCP1 and control cells. Importantly, overexpression of CDCP1 increased heterodimerization of HER2/HER3 and HER2/EGFR in normal and serum-depleted media conditions, in the absence of heregulin or EGF (Figure S5E). Similar results were obtained in SKBr3-CDCP1 cells (data not shown). Taken together, these findings demonstrate that CDCP1 binds to HER2, enhancing HER2 dimerization and activity in a ligand-independent manner.

We next evaluated which CDCP1 domains are important for the HER2 interaction. For this purpose, we transiently transfected HEK293T-HER2 cells with vectors expressing the ICD or ECD of CDCP1 (Figure 5D). Interestingly, immunoprecipitation analysis showed that only ICD-CDCP1 interacts with HER2 (Figure 5E). Further, we sought to determine whether the non-receptor tyrosine kinase c-SRC (SRC) was required for CDCP1/HER2 binding. Recent studies demonstrated that CDCP1 is a crucial binding partner of SRC (Benes et al., 2005, 2012). Moreover, SRC interacts with HER2 in breast cancer and thus plays an important role during breast cancer progression (Xu et al., 2007). Therefore, we investigated whether CDCP1 might interact with HER2 indirectly via SRC. We transfected HEK293T-HER2 cells with control vector, CDCP1, and delta-CDCP1, a mutant form of CDCP1 that does not bind to SRC (Benes et al., 2005, 2012). Importantly, immunoprecipitation of HER2 followed by immunoblotting of CDCP1 revealed that both CDCP1 and delta-CDCP1 bind to HER2 (Figure 5F, right panel). Although delta-CDCP1 did not affect HER2 binding, it did affect the phosphorylation of HER2 in different cell lines as compared with CDCP1 (Figures S6A and S6B). Together, these findings demonstrate that SRC is not required for CDCP1/HER2 binding, but is needed for HER2 phosphorylation (Spassov et al., 2011). Consistently, overexpression of delta-CDCP1 did not increase cell migration as compared with wild-type (WT)-CDCP1 in SKBr3 cells (Figure S6C).

CDCP1 Enhances the SRC-HER2 Interaction, Promoting Resistance to Trastuzumab

SRC is known to phosphorylate HER2 at the Y877 residue (Xu et al., 2007). Moreover, phosphorylation of HER2 at Y877 is implicated in HER2 activity (Parsons and Parsons, 2004). Therefore, we investigated whether HER2 was phosphorylated in this residue in CDCP1-overexpressing cells, and found that HER2 was strongly phosphorylated at Y877 (Figure 6A). This was mirrored by a concomitant phosphorylation of HER2 at Y1248 and EGFR (Y1068) in two different cell lines overexpressing CDCP1 (Figure 6B). Notably, silencing CDCP1 in MDA-MB361 cells decreased SRC activity and subsequently HER2 phosphorylation at both Y877 and Y1248 (Figure 6C). To explore whether inactivation of SRC might influence HER2 activity in CDCP1-overexpressing cells, we next downregulated SRC by using two different shRNAs. Immunoblot analysis showed that

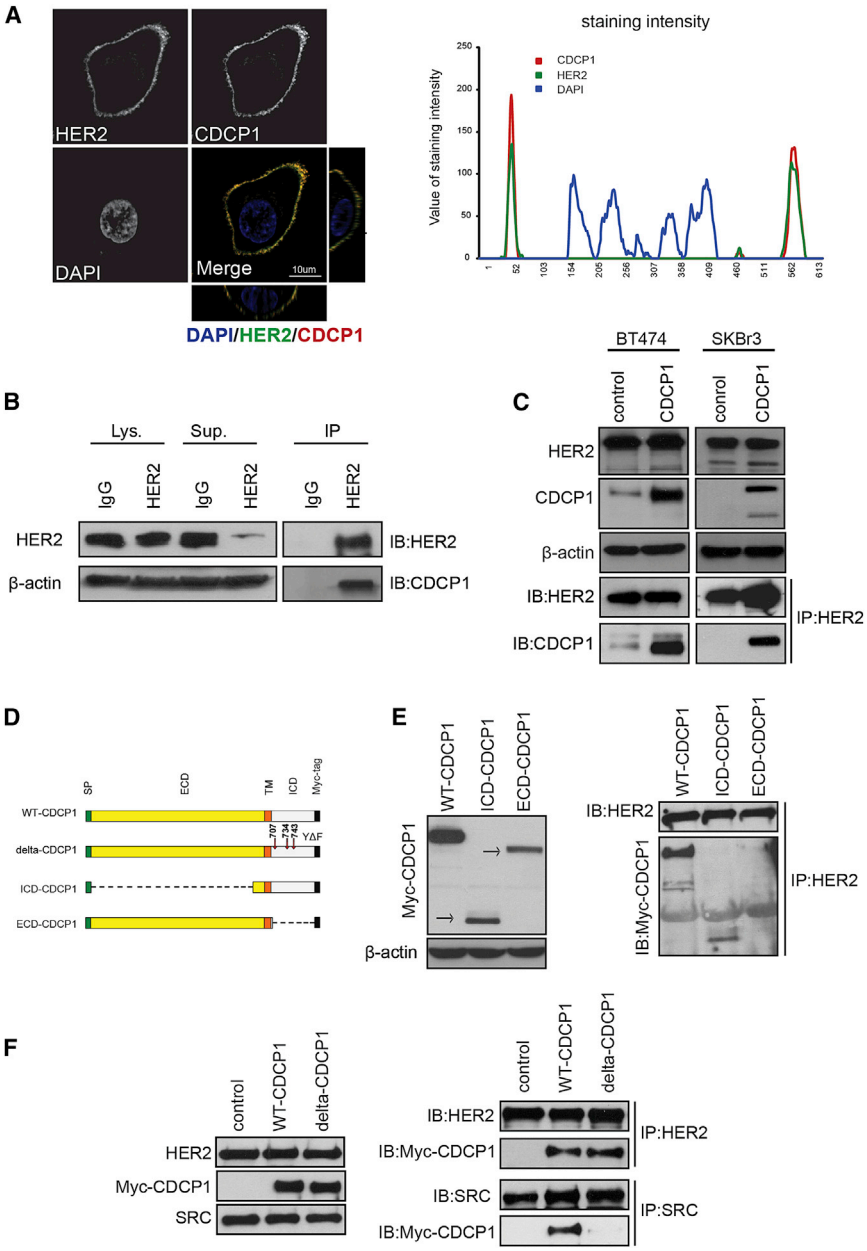

Figure 5. CDCP1 Interacts with HER2

(A) Left: confocal images of immunofluorescence staining for HER2 and CDCP1 of MDA-MB361 cells that overexpress CDCP1. Scale bar, 10 μm. Right: graph showing the membrane intensity of CDCP1 and HER2 staining.

(B) Immunoblot analysis of MDA-MB361 from total cell lysate (Lys.) or supernatant (Sup.) upon immunoprecipitation (IP) analysis using HER2 antibody. Right: HER2 IP analysis followed by immunoblot analysis for HER2 and CDCP1.

(C) Top panel: immunoblot analysis of BT474 and SKBr3 cells expressing control or CDCP1 vectors, showing the total lysate levels of HER2, CDCP1, and β-actin. Bottom panel: HER2 IP followed by immunoblot analysis for HER2 and CDCP1.

(D) Schematic representation of the WT-CDCP1 and mutant delta-CDCP1 intracellular domain (ICD) and extracellular domain (ECD) constructs. SP, signal peptide; TM, transmembrane domain.

(E) Left: immunoblot analysis of HEK293T-HER2 cells expressing WT-CDCP1, ICD-CDCP1, or ECD-CDCP1. Right: HER2 IP followed by immunoblot analysis for HER2 and Myc-CDCP1.

(F) Left: immunoblot analysis of HEK293T-HER2 cells expressing control, WT-CDCP1, or delta-CDCP1. Right: HER2 IP followed by immunoblot analysis for HER2 and Myc-CDCP1 (top panel), and SRC IP followed by immunoblot analysis for SRC and Myc-CDCP1 (bottom panel).

See also Figures S5 and S6.

silencing of SRC decreased p-HER2 at the Y877 and Y1248 residues (Figure 6D). These results showed that CDCP1 plays a crucial role in mediating the crosstalk between SRC and HER2. To validate these findings, we investigated whether CDCP1 could promote a physical interaction between SRC and HER2 at the cell membrane. For this purpose, we performed immunoprecipitation of HER2 followed by immunoblot of HER2, CDCP1, and SRC. Notably, in cells overexpressing CDCP1, we found more SRC bound to HER2 than in control cells, demonstrating that CDCP1 promotes the formation of a tertiary complex favoring the SRC-HER2 interaction (Figure 6E). Further immunofluorescence in SkBr3 confirmed a strong localization of SRC at the cell membrane only in cells overexpressing CDCP1 (Figure 6F). Interestingly, downregulation of CDCP1 abrogated the phosphorylation of SRC in HER2-overexpressing cells, suggesting that in this context, HER2 might have mediated SRC phosphorylation through CDCP1 (Figure 6G). We further confirmed this finding by using a HER2 kinase inhibitor in cells expressing CDCP1 or control vector. Indeed, we found that inhibition of HER2 activity abolished the SRC phosphorylation induced by CDCP1 expression (Figure S6D).

To assess the functional relevance of the CDCP1/SRC/HER2 complex, we sought to determine whether CDCP1 overexpression could drive resistance to trastuzumab. Recent studies have demonstrated that SRC activation plays an essential role in trastuzumab resistance in breast cancer (Zhang et al., 2011). In agreement with those studies, we found that HER2-positive tumor cells overexpressing CDCP1 were less responsive to trastuzumab than control cells (Figure 6H, left panel). Further analysis showed that trastuzumab reduced HER3 activity in both cell lines, whereas in CDCP1 cells SRC activity remained high (Figure 6H, right panel). To validate these findings in vivo, we orthotopically injected BT474 overexpressing CDCP1 and control cells into the mammary glands of immunodeficient SCID mice. At tumor onset, the mice were treated with trastuzumab weekly and tumor

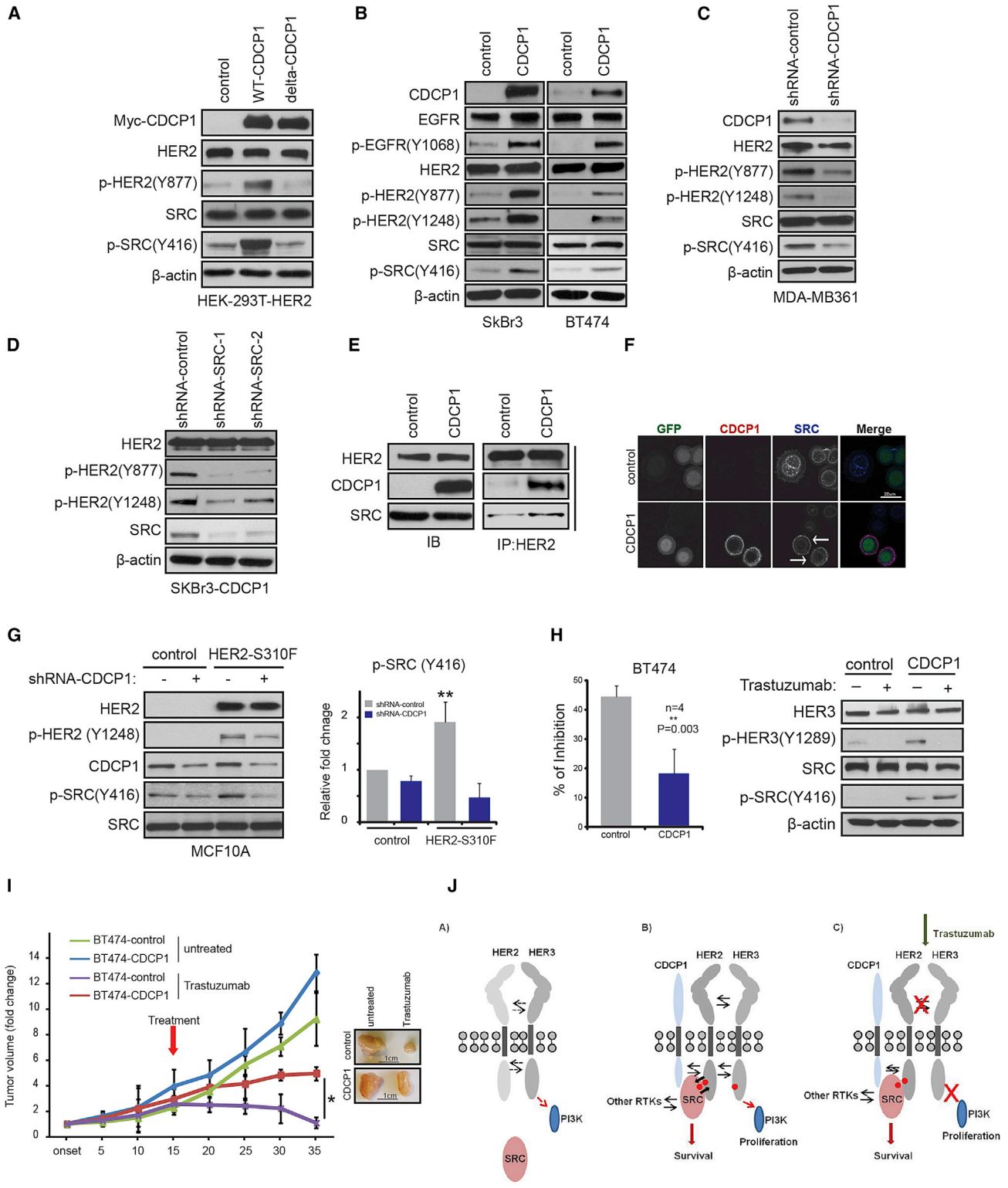

Figure 6. CDCP1 Enhances HER2 Activity through SRC, and CDCP1 Overexpression Confers Resistance to Trastuzumab Both In Vitro and In Vivo

(A) Immunoblot analysis of HER2, p-HER2 (Y877), SRC, and p-SRC (Y416) of HEK293T HER2 cells expressing control, WT-CDCP1, or delta-CDCP1.

(B) Immunoblot analysis of EGFR p-EGFR (Y1068), HER2, p-HER2 (Y877), p-HER2 (Y1248) SRC, and p-SRC (Y416) of SkBr3 and BT474 cell lysates over-expressing CDCP1 and control vectors.

(legend continued on next page)

size was measured for up to 35 days. Importantly, CDCP1-overexpressing tumors were significantly less responsive to trastuzumab compared with the control groups (Figure 6I). Collectively, these results showed that CDCP1 enhances the SRC-HER2 interaction, driving trastuzumab resistance in vitro and in vivo. Therefore, we hypothesized that targeting both SRC and HER2 could be an efficient strategy to overcome trastuzumab resistance driven by CDCP1 overexpression. To test this hypothesis, we orthotopically injected BT474-overexpressing CDCP1 in the mammary glands of immunodeficient SCID mice. We then treated established CDCP1-HER2-positive breast tumors with trastuzumab alone or in combination with an SRC inhibitor (saracatinib) for 30 days. Intriguingly, only the combination of trastuzumab and saracatinib significantly reduced the tumor volume in vivo, overcoming trastuzumab resistance (Figure S6E). This finding indicates that trastuzumab and saracatinib should be used in combination as a therapy for HER2-positive breast cancer patients possessing CDCP1 overexpression.

DISCUSSION

Despite the clinical relevance of HER2 in breast tumorigenesis, accumulating experimental evidence in vivo demonstrates that although HER2 overexpression initiates tumorigenesis, it is not sufficient to drive the progression of benign tumors into invasive and metastatic breast cancers (Andrechek et al., 2003; Lu et al., 2009; Muthuswamy et al., 2001). It is not known which additional genetic alterations may cooperate with HER2 to progress to life-threatening breast tumors. Here, we identified CDCP1 as a regulator of HER2-driven tumorigenesis. By examining two TMAs including more than 200 breast cancer cases, we found that CDCP1 was frequently co-overexpressed with HER2 in metastatic breast tumors. Importantly, HER2/CDCP1 co-overexpression in tumors was associated with poor prognosis in two large gene expression data sets of breast cancer that included more than 1,500 breast cancer samples. Although CDCP1 overexpression in cancer was previously reported in different tumor types, the mechanism behind CDCP1 function in cancer remains unknown (Casar et al., 2012, 2014; Kollmorgen et al., 2013; Liu et al., 2011; Uekita et al., 2008; Wortmann et al., 2009). A previous study demonstrated that CDCP1 overexpression enhances the metastatic potential of several cancer cell lines in vivo and promotes tumorigenesis driven by HIF-2α overexpression in kidney cancer (Emerling et al., 2013). In contrast to these findings, a recent report suggested that CDCP1 plays a tumor-suppressive role in tumor development and outgrowth (Spassov et al., 2013). Our findings show that CDCP1 cooperates with HER2 to drive breast tumorigenesis, which is in agreement with a well-documented oncogenic function of CDCP1 in cancer and supports further assessment of CDCP1 status as a useful prognostic marker in HER2-positive breast cancer patients. Indeed, our findings demonstrate that CDCP1 overexpression cooperates with HER2 in driving colony formation, tumor cell migration, tumor growth, and trastuzumab resistance in vivo. In line with these findings, we also observed that downregulation of CDCP1 in tumor cell lines harboring HER2 active mutations abolished migration, invasion, and tumorigenesis in vivo. Moreover, CDCP1 overexpression accelerated tumor progression in HER2-positive breast cancer cell lines. Mechanistically, we also dissected the role of CDCP1 in HER2 signaling and obtained both in vitro and in vivo evidence that CDCP1 overexpression enhanced HER2 phosphorylation and activation. We found that CDCP1 overexpression increased the phosphorylation of HER2 at Y1248, activating both the PI3K/AKT and MAPK/ERK pathways in different HER2-positive breast cancer cell lines. Using immunofluorescence, we also showed that cells expressing CDCP1 had higher p-HER2 (Y1248) and p-HER3 (Y1289) levels on their cell membrane compared with control cells, and that this phenotype could be reversed by the HER2 kinase inhibitor CP-724714. Finally, IHC analysis of p-HER2 (Y1248) showed that CDCP1 overexpression also enhanced p-HER2 staining in vivo. Through a series of immunoprecipitation experiments in cells expressing both exogenous and endogenous CDCP1 protein levels, we obtained evidence of a physical interaction between CDCP1 and HER2 that promotes the recruitment of SRC at the membrane. Importantly, we showed that CDCP1 interacts with HER2 through its ICD and that this interaction (HER2-CDCP1) is not mediated via SRC. However, we found that only WT-CDCP1 enhanced HER2 phosphorylation and cell migration, and an unphosphorylated form of CDCP1 (delta-CDCP1) that

(C) Immunoblot analysis of HER2, p-HER2 (Y877), p-HER2 (Y1248) SRC, and p-SRC (Y416) of MDA-MB361 lysates expressing doxycycline-inducible shRNA-control or shRNA-CDCP1.
(D) Immunoblot analysis of HER2, p-HER2 (Y877), p-HER2 (Y1248), and SRC of SkBr3 cell lysates overexpressing CDCP1.
(E) Left: immunoblot analysis of HER2, CDCP1, and SRC of BT474 control and CDCP1 total lysate. Right: HER2 IP followed by immunoblot analysis for HER2, CDCP1, and SRC.
(F) Confocal images of immunofluorescence for HER2, CDCP1, or SRC staining of SKBr3-control and SKBr3-CDCP1 cells. Scale bar, 20 μm.
(G) Left: immunoblot analysis of HER2, p-HER2 (Y1248), CDCP1, p-SRC (Y416), and SRC of MCF10A-control and MCF10A-HER2-S310F cells expressing doxycycline-inducible shRNA-control or shRNA-CDCP1. Right: bar graph represents the fold change of p-SRC (Y416) normalized to the total protein ± SD ($n = 3$, $^{**}p < 0.01$).
(H) Left: cell proliferation assay comparing the cell growth inhibition of BT474 expressing control and CDCP1 upon treatment with freshly added trastuzumab for 4 days (1 μg/ml; $n = 4$, $^{**}p < 0.01$). Right: immunoblot analysis of HER3, p-HER3 (Y1289), SRC, and p-SRC (Y416) of BT474-control and BT474-CDCP1 cells treated with trastuzumab compared with untreated cells.
(I) Left: Fold change of the tumor volume (mm^3) of BT474-control and BT474-CDCP1 upon treatment with trastuzumab for 35 days (3 mg/kg). Right: representative BT474 orthotopic xenograft tumors. Scale bar, 1 cm.
(J) Model of the CDCP1/SRC/HER2 complex. CDCP1 enhances the activation of HER2 by recruiting SRC on the membrane, thereby forming a complex with HER2. A, inactive pathway in the physiological condition; B, CDCP1 activates HER2 through SRC activation; C, trastuzumab blocks HER2/HER3 heterodimer and PI3K pathway, but not the CDCP1/SRC/HER2 axis, resulting in trastuzumab resistance.
See also Figure S6.

cannot bind to SRC had no effect on HER2 activation. This finding is in agreement with previous reports demonstrating that phosphorylation of CDCP1 and SRC is required to promote cell migration and tumorigenesis driven by CDCP1 (Liu et al., 2011). Of note, we demonstrated that CDCP1 recruits SRC to the membrane, thereby facilitating its interaction with HER2. As a result of this interaction, SRC promotes the phosphorylation and activation of HER2, which in turn sustains the phosphorylation of SRC. This positive-feedback loop triggers cell migration, invasion, tumorigenesis, and resistance to trastuzumab in vivo. Recent studies demonstrated that the two major mechanisms that drive trastuzumab resistance in breast tumors depend on SRC activation (Zhang et al., 2011). In de novo trastuzumab resistance, SRC activation is mediated by loss of the tumor suppressor PTEN, which can dephosphorylate SRC (Nagata et al., 2004). Upon acquiring resistance to trastuzumab (Moulder et al., 2001; Ritter et al., 2007), tumor cells promote the activation of SRC through an unspecified mechanism involving the activation of different RTKs (Zhang et al., 2011). Our findings complement these previous results by demonstrating that CDCP1 overexpression in tumor cells can promote the activation of HER2 by recruiting SRC. Indeed, we found that cells overexpressing CDCP1 are less responsive to trastuzumab. These data are in agreement with previous reports showing that CDCP1 is frequently upregulated in cells that acquire trastuzumab resistance (Boyer et al., 2013), thus providing a new mechanism for acquired resistance to trastuzumab. Our data also provide a proof of principle that targeting CDCP1 can interfere with HER2 signaling in vivo. Thus, these findings pave the way for future development of compounds that target CDCP1 for the treatment of HER2-positive breast cancer patients. Given that CDCP1 antibodies have been developed for cancer therapy (Kollmorgen et al., 2013; Siva et al., 2008), it is intriguing to hypothesize that such compounds may overcome trastuzumab resistance. Although further studies are needed to validate the efficacy of CDCP1-targeting compounds in HER2-positive breast cancer, our experimental model provides a rationale for testing CDCP1 antibodies and small-molecule inhibitors in HER2-positive breast cancers.

EXPERIMENTAL PROCEDURES

Gene Expression Profile Analysis

Total RNA was isolated with TRIzol (Life Technologies) according to the manufacturer's instructions. Samples were processed using the HumanHT-12 v4 Expression BeadChip kit (Illumina) according to the manufacturer's protocol. Arrays were read on an Illumina HiScanSQ system and signal intensity was extracted using quantile normalization. Differential expression analysis between SKBr3 cells overexpressing CDCP1 and control SKBr3 cells was done using Rank Products (Breitling et al., 2004), which is a test that differs from many other techniques in that it does not apply a sophisticated statistical model, but rather employs the calculation of rank products, resulting in a faster and simpler method. Using a corrected p value (false discovery rate [FDR]) minimum threshold of 0.25, we obtained signatures of 1,168 overexpressed and 1,275 underexpressed Illumina probes in SKBr3-CDCP1 samples (versus SKBr3-control). We performed enrichment analysis of GO terms after uploading selected probe-set identifiers into the DAVID Functional Annotation web tool, which computes enrichment of GO biological process (GOBP) terms (Dennis et al., 2003). GSEA was used to analyze the enrichment of the c2.cgp collection of gene sets from MSigDB (http://www.broadinstitute.org/gsea/msigdb/index.jsp) comparing SKBr3-CDCP1 and SKBR3 cells (Subramanian et al., 2005). We used the Oncomine Gene Expression Signatures database to search for significant overlapping of our signatures with human clinical breast cancer samples (Rhodes et al., 2004). Significance was determined using Fisher's exact test (odds ratio > 1.3; $p < 0.01$). Genes overexpressed or underexpressed in SKBr3-CDCP1 cells were loaded into the Oncomine database. We searched for overlaps using different filtering criteria ("Molecular Subtype: Biomarker" and "Clinical Outcome") based on the type of human cancer comparison performed. The CEL files have been deposited in the GEO repository (GSE67019).

HER2 and CDCP1 mRNA Expression in Human Breast Cancer Data Sets

Expression values for CDCP1 were downloaded for the Finak, TCGA, Curtis, and Kao breast cancer data sets from the Oncomine database. ERBB2 status was used as described in the Kao and Curtis data sets (Curtis et al., 2012; Kao et al., 2011). Differential expression analysis between sample groups was calculated using the t test. For Kaplan-Meier curves, patients were separated into four groups by considering both the published ERBB2 status and CDCP1 expression. For CDCP1, patients were stratified into two groups (high expression and low expression) depending on a ranking based on CDCP1 mRNA decreasing levels. Statistical analysis was done using Stata 12.1 software.

Animal Experiments

All mice were maintained under specific-pathogen-free conditions in the animal facilities of the Institute for Research in Biomedicine, and experiments were performed according to state guidelines and approved by the local ethics committee (TI-13-2013 and 5/2011). For orthotropic injection, 1×10^6 MCF7, BT474, and MCF10ANeuNT cells expressing HER2 were suspended in 100 μl of a 1:1 mixture of basement membrane matrix phenol red-free (BD Biosciences) and PBS, and injected into the mammary gland. Trastuzumab was administrated once a week at a concentration of 3 mg/kg. Trastuzumab (3 mg/kg) was given intraperitoneally (i.p.) twice a week. Saracatinib was given daily at a dose of 25 mg/kg in vehicle (0.5% hydroxypropylmethylcellulose with 0.1% Tween 20) via oral gavage.

Statistical Analysis

Data analysis was performed using a two-tailed unpaired Student's t test. Values are expressed as mean ± SEM or ± SD (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

ACCESSION NUMBERS

The GEO accession number for the gene expression profiles of SKBr3-CDCP1 and control cells reported in this paper is GSE67019.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.03.044.

AUTHOR CONTRIBUTIONS

A. Alimonti, A. Alajati, and I.G. designed and guided the research and wrote the manuscript. A. Alajati and I.G. performed all of the experiments. R.G.-E. performed bioinformatics analysis. S.P. performed immunohistochemical experiments. M.S. performed histopathological analysis. T.B. performed experiments. E.M. established and carried out fluorescence microscopy. F.M. analyzed the human data. A.R. and F.B. performed gene expression analysis and analyzed the data. C.C. interpreted the data. A. Alajati and I.G. are co-first authors.

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