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Chromatin Modifications Sequentially Enhance ErbB2 Expression in ErbB2-Positive Breast Cancers

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SUMMARY

ErbB2 gene amplification occurs in 20%–25% of breast cancers, and its therapeutic targeting has markedly improved survival of patients with breast cancer in the adjuvant setting. However, resistance to these therapies can develop. Because epigenetic mechanisms can importantly influence oncogene expression and be druggable as well, we investigated histone modifications that influence ErbB2 overexpression, independent of gene amplification. We demonstrate here that ErbB2-overexpressing breast carcinomas acquire the H3K4me3 mark on the *erbB2* promoter and that receptor-amplified tumors further acquire the H3K9ac mark, which is dependent on H3K4me3 mark acquisition. Targeting WD repeat domain 5 (Wdr5), which is absolutely required for H3K4me3 enrichment, decreased ErbB2 overexpression, associated with a decrease in the H3K4me3 mark on the *erbB2* promoter. Of note, Wdr5 silencing cooperated with trastuzumab or chemotherapy in specifically inhibiting the growth of ErbB2-positive breast tumor cells. Thus, our studies illuminate epigenetic steps in the selection for ErbB2 activation.

INTRODUCTION

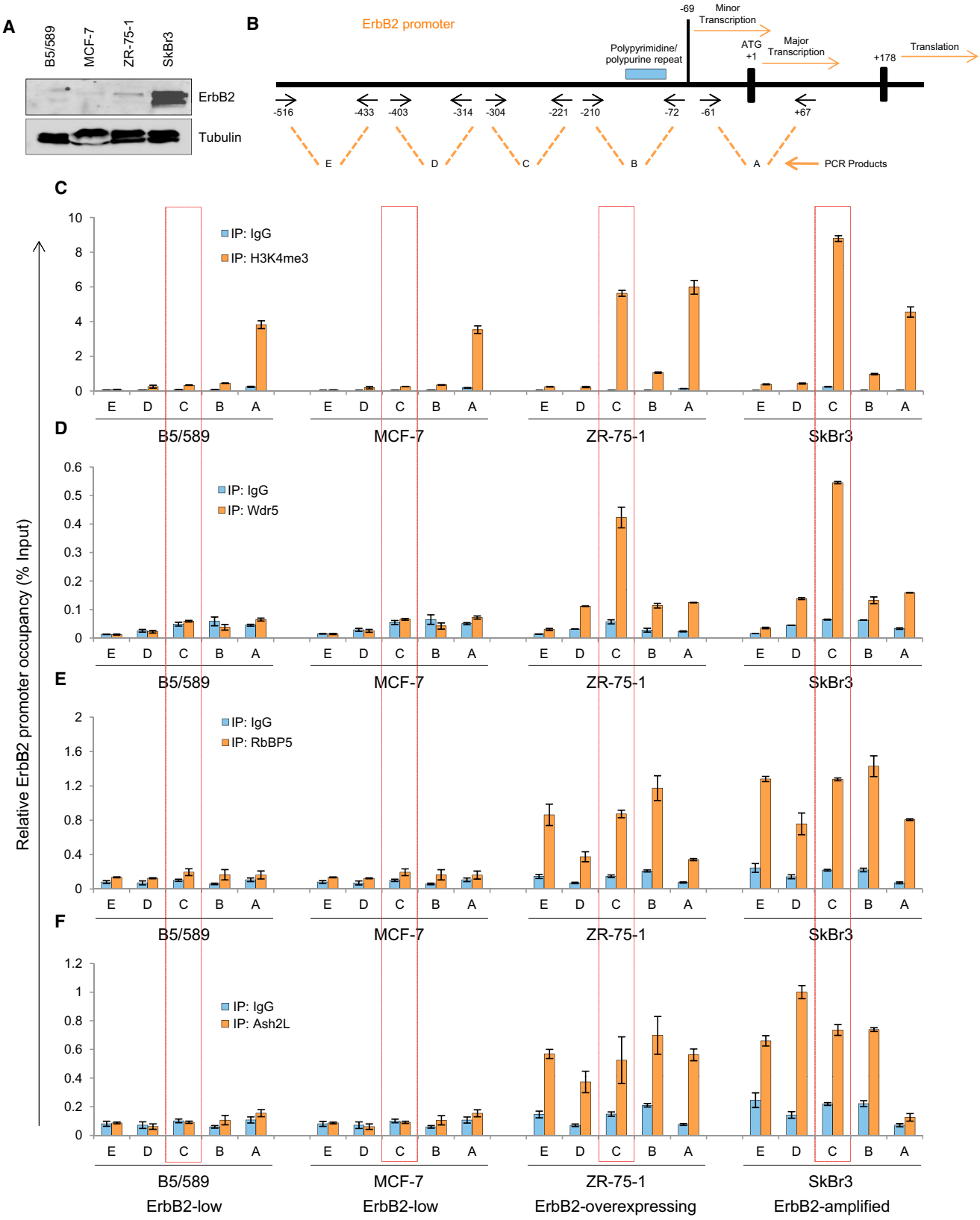
ErbB2 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2; HER2/neu) is a member of the epidermal growth factor receptor family of receptor tyrosine kinases (Dougall et al., 1994) and mediates critical signaling functions in normal and malignant breast epithelial cells (Yarden and Sliwkowski, 2001). ErbB2 gene amplification, associated with overexpression, occurs in 20%–25% of breast cancers (Slamon et al., 2011) and initially correlated with an aggressive clinical phenotype, including high-grade tumors, increased growth rate, early systemic metastasis, and decreased rate of disease-free and overall survival (Slamon et al., 1987, 1989). Targeting ErbB2 by trastuzumab or small molecule *erbB2* kinase inhibitors (Carter et al., 1992; Geyer et al., 2006) has markedly improved survival of such patients in the adjuvant setting (Piccart-Gebhart et al., 2005; Robert et al., 2006; Slamon et al., 2001). However, the majority of patients with metastatic breast cancer who initially respond to trastuzumab develop resistance within 1 year of treatment initiation, and in the adjuvant setting 15% of patients still relapse despite trastuzumab-based therapy (Nahta and Esteva, 2006). Previous studies have indicated that *erbB2*-amplified human breast cancers also exhibit higher expression levels per gene copy than would be expected (Kraus et al., 1987), as do *erbB2*-amplified tumors in a mouse knockin model (Andrechek et al., 2003), although the mechanistic basis is not yet known.

Cancers show aberrant epigenetic regulation, including global changes in DNA methylation and altered histone modifications and histone variants (Arrowsmith et al., 2012; Esteller, 2007; Rodríguez-Paredes and Esteller, 2011; Vardabasso et al., 2013). Because epigenetic mechanisms can importantly influence gene expression (Peltomäki, 2012; Rodríguez-Paredes and Esteller, 2011) and be druggable as well (Arrowsmith et al., 2012; Delmore et al., 2011; Zuber et al., 2011), we investigated histone modifications that influence ErbB2 overexpression, independent of gene amplification. We demonstrate that ErbB2-overexpressing breast carcinomas acquire the histone H3 lysine 4 trimethylated (H3K4me3) mark on the *erbB2* promoter and that receptor-amplified tumors additionally acquire the H3K9ac mark. We demonstrate that approaches that interfere with H3K4me3 mark enrichment on the *erbB2* promoter cooperate with trastuzumab or chemotherapy in specifically inhibiting the proliferation of ErbB2-positive breast tumor cells. These studies identify epigenetic mechanisms in addition to the known genetic alterations that select for increased ErbB2 expression in ErbB2-positive breast cancers.

RESULTS

ErbB2-Amplified and ErbB2-Overexpressing Cells, but Not ErbB2-Low Cells, Exhibit H3K4me3 Enrichment on the *erbB2* Promoter

Previous studies have indicated that ErbB2-overexpressing breast tumor cells exhibit 4- to 8-fold higher and ErbB2-amplified



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breast tumor cells exhibit 64- to 128-fold higher ErbB2 RNA expression levels than that observed in ErbB2-low breast tumor cells (Kraus et al., 1987). We analyzed ErbB2-amplified, overexpressing breast cancer cells (SkBr3 and BT-474; referred to as "ErbB2 amplified"); ErbB2-nonamplified, overexpressing breast cancer cells (ZR-75-1 and MDA-MB-175 VII; referred to as "ErbB2-overexpressing"); and ErbB2 nonamplified, low-expressing or undetectably expressing breast cancer (MCF-7 and BT-20) or immortalized human breast epithelial (B5/589) cells (referred to as "ErbB2-low"). We confirmed these overexpression levels and that the amplified tumor cells exhibited a further increase in expression per gene copy (Figure 1A; Table S1). In view of promising new approaches to target epigenetic aberrations in cancer (Arrowsmith et al., 2012; Delmore et al., 2011; Filippakopoulos et al., 2010; Peltomäki, 2012; Rodríguez-Paredes and Esteller, 2011; Zuber et al., 2011), we tested for local histone modifications near the *erbB2* promoter that might contribute to increased ErbB2 expression in these tumor cells. We divided the promoter from −516 bp to +67 bp into five regions (Figure 1B) and studied the relative abundance of histone marks by chromatin immunoprecipitation (ChIP) in all of these cells. We identified the H3K4me3 mark, an established histone mark for active transcription (Santos-Rosa et al., 2002), in region "C" (−304 bp to −221 bp), both in ErbB2-overexpressing and ErbB2-amplified cells, but not in ErbB2-low cells (Figures 1C and S1A). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and myogenic differentiation 1 (MyoD) transcription start sites (TSSs) served as positive and negative controls for H3K4me3 ChIP analysis (Figure S2). We did not detect histone H3 lysine 9 trimethylated (H3K9me3) or histone H3 lysine 27 trimethylated (H3K27me3) repressive chromatin marks on the *erbB2* promoter in any of these cells (data not shown), indicating the absence of a bivalent chromatin conformation (Fisher and Fisher, 2011) or evidence of promoter DNA methylation, even though the *erbB2* promoter has a CpG-rich island (Figures S3A and S3B).

H3K4me3 Enrichment on the *erbB2* Promoter Is Required for ErbB2 Overexpression

The H3K4 methyltransferase complex contains four structural components (WD repeat domain 5 [Wdr5], RbBP5, Ash2L, and DPY-30) in addition to a catalytic subunit (Dou et al., 2006; Han et al., 2006; Steward et al., 2006; Trievel and Shilatifard, 2009). In agreement with our H3K4me3 ChIP data, we observed *erbB2* promoter occupancy by Wdr5 (Figures 1D and S1B), as well as by RbBP5 (Figures 1E and S1C) and Ash2L (Figures 1F and S1D), in ErbB2-overexpressing and ErbB2-amplified cells, but not in ErbB2-low cells. Gapdh TSS and MyoD TSS were used as positive and negative controls for these ChIP assays (Figure S2). Wdr5 is a WD40 repeat protein, which functions to target dimethylated H3K4 (H3K4me2) for H3K4 trimethylation (H3K4me3) (Dou et al., 2005; Patel et al., 2009; Wysocka et al., 2005). Recent in vitro studies also have indicated that Wdr5 is unable to discriminate between H3K4 methylation states (Couture et al., 2006; Ruthenburg et al., 2006). However, H3K4me3 enrichment is absolutely correlated with Wdr5 promoter occupancy (Dou et al., 2006; Patel et al., 2009), and loss of Wdr5 affects global H3K4 trimethylation in vivo (Ang et al., 2011; Wysocka et al., 2005).

To analyze the role of Wdr5 in ErbB2 expression, we generated B5/589, ZR-75-1, and SkBr3 cells stably expressing doxycycline-inducible Wdr5 small hairpin RNA (shRNA). Doxycycline-inducible GFP shRNA was used as a negative control in all shRNA experiments to test for off-target effects of doxycycline and nonspecific shRNA effects. Addition of doxycycline to the culture medium led to a decrease in Wdr5 expression in shWdr5-expressing cells, but not in shGFP-expressing cells, as measured at mRNA (Figures 2A and 2C) and protein (Figures 2B, 2D, and S4A) levels, resulting in reduced H3K4me3 enrichment on the *erbB2* promoter both in ErbB2-overexpressing (ZR-75-1) and *erbB2*-amplified (SkBr3) cells (Figures S4B and S4C). Downregulation of Wdr5 specifically induced in each case reduced ErbB2 expression as measured at both mRNA (Figures 2A and 2C) and protein (Figures 2B and 2D) levels. To further confirm the specificity of the Wdr5 shRNA, we generated SkBr3 cells stably overexpressing Wdr5 open reading frame (ORF) in the background of inducible Wdr5 silencing. Whereas shWdr5 sequence 1 targets the Wdr5-ORF, shWdr5 sequence 2 targets the 3′ UTR. In SkBr3 vector control cells, doxycycline induction of shWdr5-Seq 1 or shWdr5-Seq 2 reduced endogenous Wdr5 mRNA and protein levels (Figures S5A and S5B; data not shown). As expected, in SkBr3 cells overexpressing Wdr5-ORF, doxycycline induction reduced Wdr5 levels in shWdr5-Seq 1-, but not in shWdr5-Seq 2-expressing cells. Of note, Wdr5-ORF overexpression was able to rescue ErbB2 expression in doxycycline-induced SkBr3-shWdr5-Seq 2-expressing cells (Figures S5A and S5B), but not in SkBr2-shWdr-Seq 1-expressing cells (data not shown). Further, releasing the cells from Wdr5 silencing by removing doxycycline from the culture medium resulted in restoration of Wdr5 expression and ErbB2 re-expression (Figure S5C). All of these results established the functional involvement of Wdr5 in increased ErbB2 expression.

Because Wdr5 has been shown to be involved in multiple histone methyltransferase (HMT) as well as histone

Figure 1. H3K4me3, Wdr5, RbBP5, and Ash2L Are Specifically Recruited onto the *erbB2* Promoter in ErbB2-Overexpressing and ErbB2-Amplified Tumor Cells

(A) Western blot analysis showing the ErbB2 protein expression across the cell lines.

(B) Schematic representation of the *erbB2* promoter, showing major and minor TSS (transcriptional start site) and the translational start site. The five regions used for chromatin immunoprecipitation (ChIP) are also indicated.

(C–F) ChIP analysis showing the H3K4me3 enrichment (C) and occupancy of Wdr5 (D), RbBP5 (E), and Ash2L (F) on the *erbB2* promoter in B5/589, MCF-7, ZR-75-1, and SkBr3 cells. The relative H3K4me3 enrichment or occupancy each protein over the % input is shown in the form of a bar diagram. Region "C" of the promoter, which shows the most significant difference between different cell types, is highlighted. Gapdh TSS and MyoD TSS were used as positive and negative controls, respectively, in each ChIP experiment (Figure S2). The error bars represent SEM. Each ChIP experiment was done in triplicate and repeated at least three times, and representative experimental data are shown.

See also Figures S1 and S2.

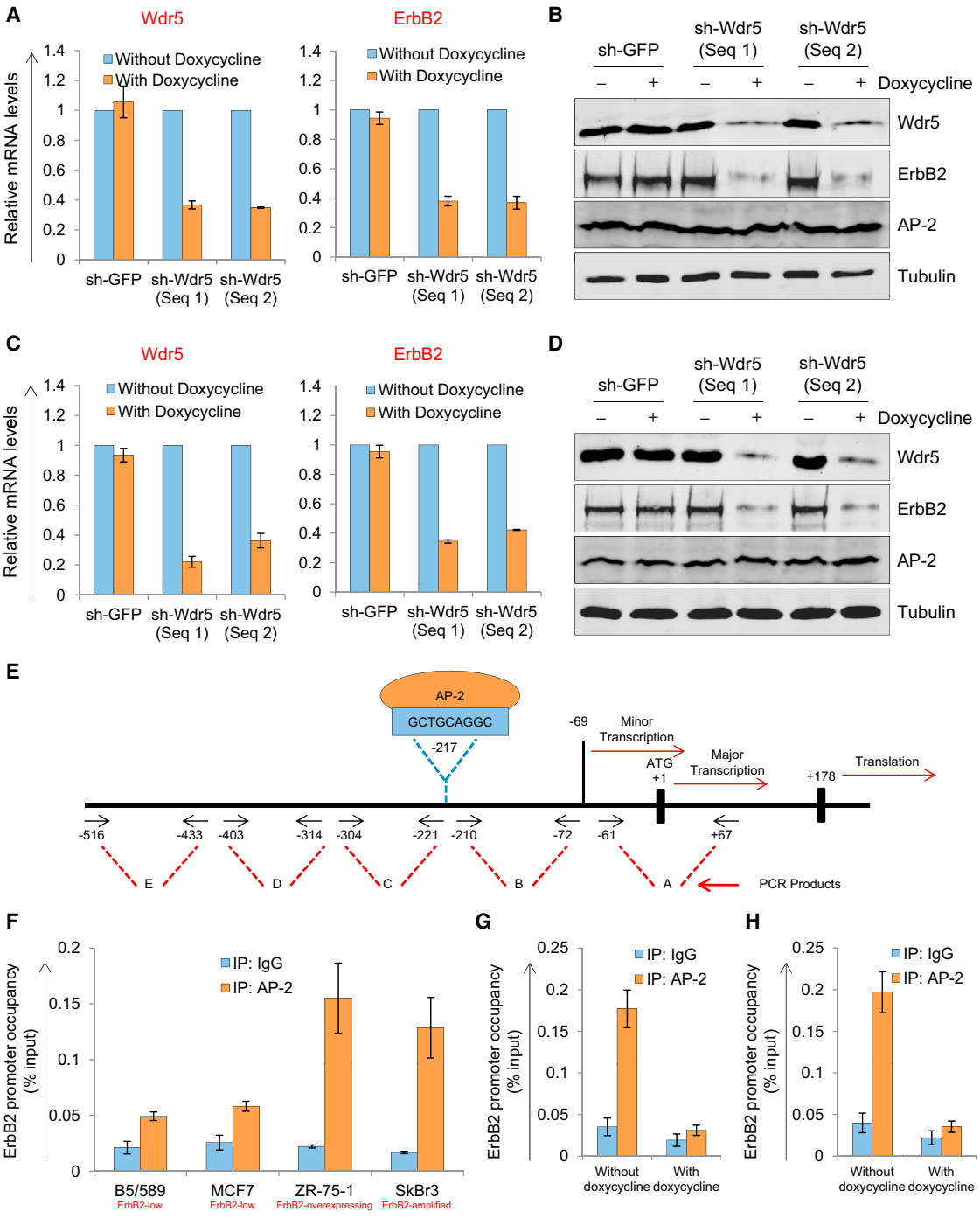

Figure 2. Silencing Wdr5 Inhibits ErbB2 Expression by Inhibiting AP-2 Recruitment, Both in ErbB2-Overexpressing and ErbB2-Amplified Cancer Cells

(A–D) Real–time quantitative PCR (A and C) and western blot analysis (B and D) of ZR-75-1 (A and B) and SkBr3 (C and D) cells stably transduced with inducible shRNA viruses and cultured in the presence of doxycycline for 48 hr.

(E) Schematic representation of the *erbB2* promoter, showing major and minor TSS and the translational start site. The five regions used for chromatin immunoprecipitation (ChIP) are also indicated. The AP-2 binding site along with the recognition sequence is shown.

(F) ChIP analysis showing the AP-2 occupancy on the *erbB2* promoter in B5/589, MCF-7, ZR-75-1, and SkBr3 cells. The target sequences (corresponding to region "C") were detected by quantitative real-time PCR analysis of eluted DNA. The relative occupancy of AP-2 over the % input is shown in the form of a bar diagram.

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acetyltransferase (HAT) complexes (Dou et al., 2005; Thompson et al., 2008), it is possible that Wdr5 knockdown affects not only H3K4 trimethylation but also other chromatin remodeling events, which might contribute to decreased ErbB2 expression. In contrast to Wdr5, Ash2L has been shown to be a specific component of an MLL (mixed lineage leukemia) complex, and Ash2L reduction leads to specific loss of H3K4me3, without altering the levels of either Wdr5 or H3K4me1 and H3K4me2 (Steward et al., 2006). To confirm the role of H3K4me3 enrichment in ErbB2 expression, we measured ErbB2 levels in cells specifically silenced for Ash2L utilizing SkBr3 cells stably expressing doxycycline-inducible Ash2L shRNA. Addition of doxycycline to the culture medium resulted in decreased Ash2L expression as measured at both mRNA (Figure S6A) and protein (Figure S6B) levels and also reduced H3K4me3 enrichment on the *erbB2* promoter (Figure S6C). In addition, downregulation of Ash2L was associated in each case with reduced ErbB2 expression as measured at both mRNA (Figure S6A) and protein (Figure S6B) levels. These results establish that H3K4me3 enrichment by an MLL complex observed on the *erbB2* promoter is essential for increased ErbB2 expression.

H3K4me3 Enrichment Enables AP-2 Recruitment on the *erbB2* Promoter

We next sought to determine whether H3K4me3 enrichment influences recruitment to the *erbB2* promoter of transcription factors that are known to regulate ErbB2 expression. The activator protein-2 (AP-2) binding site on the *erbB2* promoter was mapped to a region −217 bp upstream of the *erbB2* TSS (Bosher et al., 1995). Moreover, this site was immediately adjacent to region "C" of the *erbB2* promoter, which was positive for H3K4me3 and its readers (Figure 2E). Confirming previous findings (Bosher et al., 1995), ChIP analysis showed that AP-2 exhibited increased occupancy on the *erbB2* promoter in overexpressing and amplified tumors as compared to ErbB2-low cells (Figure 2F). Further, we observed reduced AP-2 promoter occupancy in response to Wdr5 silencing in both ErbB2-overexpressing (Figure 2G) and ErbB2-amplified cells (Figure 2H), without detectable changes in the total cellular AP-2 levels (Figures 2B and 2D). In contrast, we observed no differences in AP-2 promoter occupancy on the BCl-2 promoter, another well-established AP-2 target gene (Wajapeyee et al., 2006), across the cell lines tested (Figure S7A). Further, silencing Wdr5 did not affect AP-2 occupancy on the BCl-2 promoter (Figure S7B), even though it reduced H3K4me3 enrichment on the BCl-2 TSS (Figure S7C). Taken together, these results indicate that Wdr5 recruitment facilitates H3K4me3, which in turn favors AP-2 occupancy specifically on the *erbB2* promoter.

The *erbB2* Promoter Is H3K9 Acetylated in *erbB2*-Amplified, but Not in ErbB2-Overexpressing and ErbB2-Low, Breast Tumor Cells

Previous studies have indicated that *erbB2*-amplified breast tumor lines express more transcripts per gene copy than would be expected based on overexpression of the single-copy gene (Kraus et al., 1987). We confirmed these findings with ZR-75-1 cells, which showed a 4-fold increase in expression of ErbB2 compared to ErbB2-low MCF-7 cells. SkBr3 cells, which showed 4- to 8-fold gene amplification, overexpressed ErbB2 128-fold, rather than the expected 16- to 32-fold, compared to MCF-7 cells (Kraus et al., 1987) (Figure 1A; Table S1; Figures S11A and S11B). This discrepancy between predicted and actual levels of ErbB2 overexpression with gene amplification has been observed also in the Neu knockin mouse model, in which transcriptional upregulation of Neu mRNA is greater than can be accounted for by the 2- to 20-fold amplification of the activated *neu* allele (Andrechek et al., 2003). Thus, we reasoned that there might be additional promoter chromatin modifications in *erbB2*-amplified breast tumors. In fact, we observed histone H3 lysine 9 acetylation (H3K9ac) in ErbB2-amplified, but not in ErbB2-overexpressing or ErbB2-low cells (Figures 3A and S8A). Gapdh TSS, MyoD TSS, and ALDOA TSS were used as positive and negative controls for H3K9ac ChIP assays. Note that ALDOA (aldolase A) TSS was positive for H3K4me3 and negative for the H3K9ac histone mark (Figure S2). This modification had functional significance, shown by treatment with anacardic acid, a known HAT inhibitor (Mai et al., 2006; Rodríguez-Paredes and Esteller, 2011), which reduced ErbB2 expression levels specifically in *erbB2*-amplified cells but had no detectable effect on ErbB2-overexpressing, nonamplified cells as measured at both mRNA (Figure 3B) and protein (Figure 3C) levels.

Gcn5 Mediates H3K9 Acetylation on the *erbB2* Promoter in *erbB2*-Amplified Breast Tumor Cells

Anacardic acid has been shown to inhibit the activities of several HATs, including p300, PCAF, Tip60, and Gcn5 (Hemshekhar et al., 2011). The H3K9ac mark is written by PCAF or Gcn5 (Kouzarides, 2007), each of which has distinct as well as overlapping functions (Xu et al., 2000). Further, Gcn5-mediated H3K9ac is thought to recruit additional transcription factors leading to the formation of a more efficient transcription preinitiation complex (Koutelou et al., 2010). To identify the HAT responsible for H3K9 acetylation on the *erbB2* promoter, we generated stable cell lines expressing either doxycycline-inducible shPCAF or shGcn5. Addition of doxycycline to the culture medium reduced PCAF expression levels in SkBr3 cells as measured at both mRNA and protein (Figures S9A and S9B) levels, using two different shRNA constructs. However, PCAF silencing had no significant effect on ErbB2 expression as measured at both mRNA and protein (Figures S9A and S9B) levels. Next, we generated stable cell lines expressing doxycycline-inducible shGcn5 in both ErbB2-overexpressing ZR-75-1 and *erbB2*-amplified SkBr3 breast tumor cells. Addition of doxycycline to the culture medium reduced Gcn5 expression levels in each breast tumor line at both mRNA (Figures 3D and 3F) and protein (Figures 3E and 3G) levels, using two different shRNA constructs. Of note,

(G and H) ChIP analysis showing AP-2 occupancy on *erbB2* promoter in ZR-75-1 (G) or in SkBr3 (H) cells that were stably transduced with sh-Wdr5 (Seq 1) and cultured in the presence of doxycycline for 48 hr. The target sequences (corresponding to region "C") were detected by quantitative real-time PCR analysis of eluted DNA. The relative AP-2 promoter occupancy over the % input is shown in the form of a bar diagram. The error bars represent SEM. Each experiment was repeated at least three times, and representative experimental data are shown.

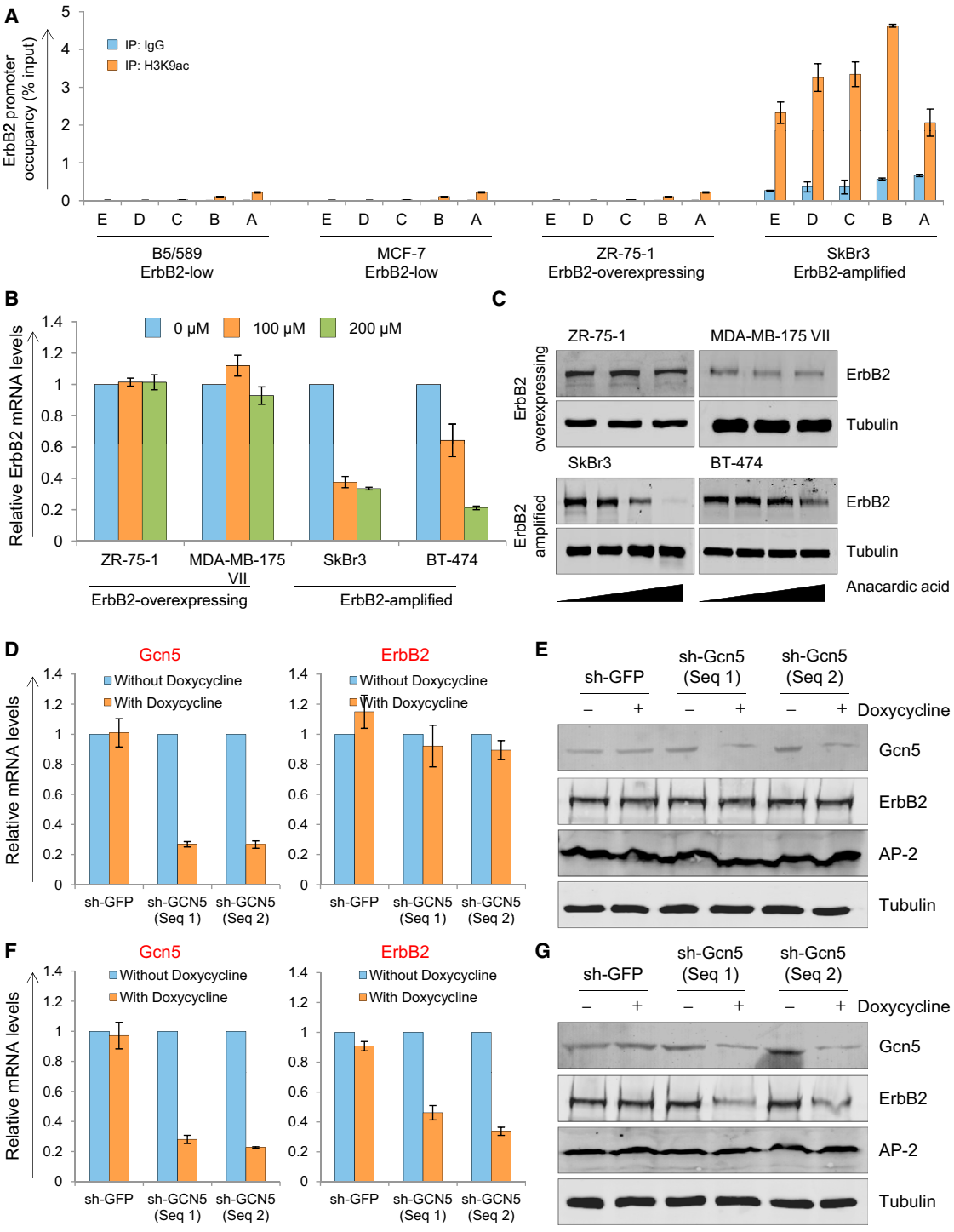

Figure 3. The *erbB2* Promoter Is H3K9 Acetylated by Gcn5 in ErbB2-Amplified Cancers Only and Not in ErbB2-Overexpressing or ErbB2-Low Cells

(A) ChIP analysis showing the H3K9ac enrichment on the *erbB2* promoter in B5/589, MCF-7, ZR-75-1 and SkBr3 cells. The relative H3K9ac enrichment over the % input is shown in the form of a bar diagram. Gapdh TSS, MyoD TSS and ALDOA TSS were used as positive and negative controls for H3K9ac ChIP assays. Note that ALDOA (aldolase A) TSS was positive for H3K4me3 and negative for H3K9ac histone mark (Figure S2).

(B and C) Quantitative real-time PCR (B) and western blot (C) analysis showing ErbB2 expression in the indicated cancer cell lines treated with either 0, 100, or 200 μM anacardic acid for 24 hr.

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Gcn5 silencing reduced ErbB2 mRNA expression specifically in *erbB2*-amplified tumor cells (Figures 3D and 3F), which correlated well with reduced protein levels (Figures 3E and 3G) and loss of H3K9ac on the *erbB2* promoter following doxycycline treatment (Figure S8B). All of these findings indicate that Gcn5-mediated H3K9ac, but not PCAF-mediated H3K9ac, was responsible for the expression of more transcripts per given gene copy in *erbB2*-amplified compared to ErbB2-overexpressing tumor lines.

We observed that Gcn5 was recruited onto the *erbB2* promoter in *erbB2*-amplified cells, but not in either ErbB2-low or in ErbB2-overexpressing cells (Figure S8C). Further, in Gcn5-silenced cells, there was no detectable change in H3K4me3 enrichment (Figure S8D) or in AP-2 promoter occupancy (Figure S8E). In contrast, Wdr5 silencing resulted in reduced H3K9ac enrichment on the *erbB2* promoter in SkBr3 cells (Figure S10). These findings argue strongly that H3K9ac enrichment on the *erbB2* promoter in ErbB2-amplified breast cancers requires the presence of the H3K4me3 mark.

Validation of Histone PTMs in ErbB2-Positive Breast Tumor Tissues

We next sought to confirm the presence of the histone posttranslational modifications (PTMs) identified in ErbB2-overexpressing and *erbB2*-amplified breast tumor lines in primary human breast cancer tissues. ChIP analysis with H3K4me3- and H3K9ac-recognizing antibodies revealed that ErbB2-overexpressing, nonamplified primary tumors showed the H3K4me3 mark, but not the H3K9ac mark, whereas *erbB2*-amplified tumor samples exhibited both marks on the *erbB2* promoter (Figures 4A and S11A). In contrast, tissue samples from ErbB2-low breast cancer patients showed neither of these histone modifications on the *erbB2* promoter (Figures 4A and S11A). In each case, the enrichment of these histone marks correlated with the ErbB2 mRNA expression levels (Figure S11B). Thus, the histone marks observed in primary breast tumors reflected those we identified in ErbB2-positive breast tumor cell lines.

ErbB2 and Grb7 Exhibit Different Mechanisms of Overexpression, Even Though They Are Coamplified and Coexpressed

Growth factor receptor bound protein-7 (Grb7) gene is commonly coamplified and overexpressed with ErbB2 in breast cancer lines and primary tumors (Stein et al., 1994). Grb7 has been shown to physically interact with ErbB2 and to enhance its signaling functions (Nadler et al., 2010; Stein et al., 1994) as well as those of several other tyrosine kinases through its SH2 domain (Pero et al., 2003). Moreover, Grb7 has been reported to contribute to tumorigenesis by the *erbB2* oncogene in mouse models (Kao and Pollack, 2006). Grb7 was not overexpressed in ErbB2-overexpressing, nonamplified tumor cells (data not shown), in agreement with recent studies (Ramsey et al., 2011). Further, silencing of either Wdr5 or Gcn5 in SkBr3 cells did not alter Grb7 expression levels as measured at both mRNA (data not shown) and protein (Figure S12) levels. These findings argue that Grb7 does not utilize these same epigenetic mechanisms to upregulate its transcription.

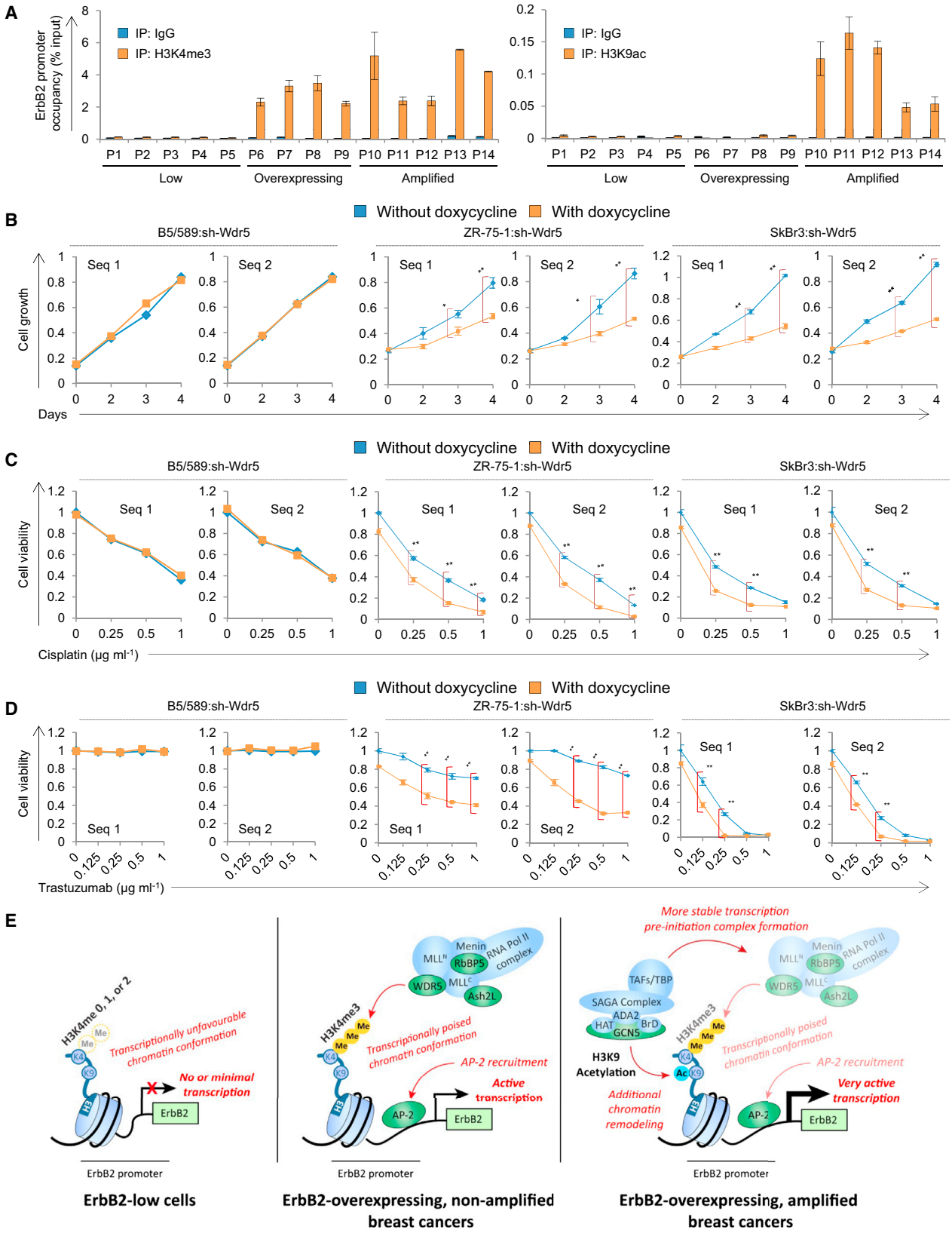
Effects of Targeting Epigenetic Proteins On Chemotherapy- and Trastuzumab-Induced Breast Cell Growth Inhibition

Next, we investigated the functional consequences of Wdr5 silencing on breast tumor cell growth. Wdr5 silencing reduced cell growth in both ErbB2-overexpressing and ErbB2-amplified tumor cells, but had no detectable effect on ErbB2-low immortalized breast epithelial cells (Figure 4B). Moreover, Wdr5 presilencing enhanced the sensitivity of both ErbB2-overexpressing and *erbB2*-amplified, but not ErbB2-low, immortalized breast cancer cells to cisplatin, a chemotherapy agent used in breast and ovarian cancer therapy (Pegram et al., 1999) (Figure 4C). Trastuzumab, a humanized ErbB2-targeting antibody that has been shown to improve progression-free survival and overall survival in breast cancer patients with *erbB2* amplification (Paik et al., 2008), reduced viability of ErbB2-amplified tumor cells in a dose-dependent manner (Figure 4D). It had a more modest effect on viability of ErbB2-overexpressing cells and no effect on ErbB2-low immortalized breast epithelial cells (Figure 4D). Presilencing of Wdr5 in combination with trastuzumab also did not affect the viability of ErbB2-low immortalized breast epithelial cells (Figure 4D), whereas Wdr5 presilencing enhanced the growth inhibitory effect of trastuzumab in *erbB2*-amplified cells (Figure 4D). Compared to *erbB2*-amplified tumor cells, ErbB2-overexpressing cells responded only modestly to trastuzumab as a single agent, but showed significantly greater growth inhibition in combination with Wrd5 presilencing as measured in short-term culture by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Figure 4D) or longer term by soft agar assay (Figure S13). These results suggest that trastuzumab in combination with Wdr5 targeting may be a promising therapeutic approach for patients with ErbB2-overexpressing breast cancer cells and potentially for ErbB2-positive breast cancers that become resistant to conventional targeted therapies.

DISCUSSION

Transgenic targeting of the overexpressed wild-type *erbB2* gene in the mouse mammary gland has been shown to select for mammary tumors with ErbB2 overexpression and the well-characterized neu mutation (Guy et al., 1992). Similarly, knocking in of the mutant form under control of the endogenous promoter leads to mammary tumors that also overexpress and amplify this gene (Andrechek et al., 2003), indicating in each case a stepwise selection for greater ErbB2 expression/activity. In humans, analogous mutations of the *erbB2* gene are generally not observed.

(D–G) Quantitative real-time PCR (D and F) and western blot (E and G) analysis of ZR-75-1 (D and E) and SkBr3 (F and G) cells stably transduced with inducible shRNA viruses and cultured in the presence of doxycycline for 48 hr. Doxycycline-inducible GFP shRNA was used as a negative control in all shRNA experiments to test for off-target effects of doxycycline and nonspecific shRNA effects. The error bars represent SEM. Each experiment was repeated at least three times, and representative experimental data areshown.
See also Figure S2.



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However, different levels of overexpression and of gene amplification/overexpression have been reported among human ErbB2-positive tumors. In the present study, we elucidated epigenetic modifications that accumulate on the *erbB2* promoter, leading to increased expression of this proto-oncogene. AP-2 is highly expressed in breast cancer cell lines overexpressing ErbB2 and positively regulates ErbB2 expression (Bosher et al., 1995, 1996; Grooteclaes et al., 1999). We showed that H3K4me3 enrichment on the *erbB2* promoter is responsible for enhanced AP-2 promoter occupancy and elevated ErbB2 expression. Our findings that the H3K4me3 mark is common to both tumor subsets indicate that overexpression and amplification are epigenetically linked. Moreover, our evidence that in *erbB2* receptor-amplified tumors H3K9ac specifically accumulates on the *erbB2* promoter associated with further enhancement of expression per gene copy is consistent with this mark occurring as the result of continued epigenetic selective pressure (Figure 4E). These newly observed epigenetic alterations take place in the context also of amplification/overexpression of Grb7, an oncogene shown here to be under independent epigenetic control. Thus, *erbB2* activation in human breast cancers can involve a number of discrete epigenetic as well as genetic alterations (Andrechek et al., 2000; Di Fiore et al., 1987; Muller et al., 1988).

MLL complexes have been shown to methylate H3K4, and Wdr5 is a conserved subunit of these complexes and has been shown to be required to maintain MLL complex integrity (Steward et al., 2006). Although Wdr5 has been reported not to discriminate mono-, di-, tri-, or unmethylated H3K4 positions (Couture et al., 2006; Ruthenburg et al., 2006), it affects global trimethylation at the same residue (Dou et al., 2006; Wysocka et al., 2005). In contrast to Wdr5, Ash2L is a specific component of H3K4me3 methyl transferase complex, and its reduction led to specific loss of H3K4me3 (Steward et al., 2006). In the present study, silencing of either Wdr5 or Ash2L abrogated H3K4me3 enrichment on the *erbB2* promoter and interfered with ErbB2 expression, establishing that this histone mark enrichment plays a major role in enhancing *erbB2* expression in *erbB2* positive breast tumor cells.

Human Gcn5 is part of the large SAGA (Spt-Ada-Gcn5 acetyl transferase) complex, which enforces gene expression patterns by acetylating histones (Koutelou et al., 2010; Rodríguez-Navarro, 2009). We detected H3K9ac on the *erbB2* promoter only in amplified tumors. Moreover, Gcn5 silencing reduced H3K9ac without affecting the H3K4me3 enrichment, observed in all ErbB2 positive tumors. In contrast, silencing Wdr5 reduced both H3K4me3 and H3K9ac marks on the *erbB2* promoter in *erbB2*-amplified tumor cells, arguing strongly that H3K9ac enrichment requires the presence of H3K4me3 mark. All these findings support the concept that ErbB2-overexpression/amplification occurs in a stepwise manner, in which ErbB2 overexpression precedes *erbB2*-amplification. Whether H3K9ac enrichment arises following gene amplification or proceeds and selects for gene amplification is not known.

Recent evidence indicates that some ErbB2-overexpressing breast tumors in the absence of gene amplification respond clinically to trastuzumab (Paik et al., 2008), and a major phase 3 clinical trial has been initiated to evaluate the efficacy of trastuzumab in combination with chemotherapy in women with breast cancers designated as HER2 overexpressed but nonamplified (as determined by all HER2 tests performed) (National Surgical Adjuvant Breast and Bowel Project NSABP-B-47; NCT01275677). Thus, it would be of interest to determine whether a subset of such tumors exhibit the H3K9ac mark, associated with high levels of ErbB2 expression and better clinical response to trastuzumab.

Cancer epigenetics has achieved the oncology mainstream, with epigenetic drug discovery showing promise in therapy (Esteller, 2007, 2008; Filippakopoulos et al., 2010; Rodríguez-Paredes and Esteller, 2011). Resistance to trastuzumab can develop via multiple mechanisms, including modification of the antibody binding site (Nahta and Esteva, 2006). Lapatinib, a small-molecule inhibitor of ErbB2 tyrosine kinase activity, is a newer ErbB2-targeting drug (Geyer et al., 2006) but can lead to acquired resistance via mutation in the *erbB2* kinase domain (Kancha et al., 2011). In short-term culture, Wdr5 knockdown did not affect ErbB2-low cell proliferation or cooperate with chemotherapy. In contrast, ErbB2 nonamplified overexpressing cells showed little or no growth inhibition with trastuzumab alone, but the combination of trastuzumab with targeting Wdr5 expression strongly inhibited colony formation in soft agar. Although the long-term effects of Wdr5 silencing need to be further explored, it is possible that agents that inhibit H3K4me3 enrichment, identified by us as playing a major role in upregulating *erbB2* transcription, may one day be applicable in targeting ErbB2-positive breast tumors.

EXPERIMENTAL PROCEDURES

Patient Samples and Gene Copy Number Verification

Tissues were received from the NCI Cooperative Human Tissue Network. Use of anonymized human tissues was approved by Mount Sinai's institutional

Figure 4. Wdr5 Silencing Cooperates with Chemotherapy- and Trastuzumab-Induced Growth Inhibition

(A) ChIP analysis showing H3K4me3 and H3K9ac enrichment on the *erbB2* promoter in breast cancer patient samples, where P1 through P14 indicate different patients. The relative H3K4me3 and H3K9ac enrichment over the % input is shown in the form of a bar diagram. See also Figure S11.

(B) Cell growth of the indicated cell lines with or without doxycycline (to induce Wdr5-shRNA) in the culture medium.

(C and D) Cell viability of the indicated cells in the absence and presence of doxycycline and with increasing doses of either Cisplatin (C) or trastuzumab (D). The error bars represent SEM. Each experiment (except A: patient ChIPs; done once) was repeated at least three times, and representative experimental data are shown. The statistical significance was calculated by Student's t test in MS Excel using two-tailed analysis. A p value <0.005 is indicated by one star, and <0.0005 is indicated by two asterisks.

(E) Schematic model showing the regulation of chromatin conformation on the *erbB2* promoter in different cancers. In ErbB2-nonamplified/overexpressing and ErbB2-amplified/overexpressing cancers, the promoter is H3K4 trimethylated, which opens chromatin structure and facilitates the recruitment of the AP-2 transcription factor, leading to ErbB2 transcription. In ErbB2-amplified/overexpressing cancers only, Gcn5 causes H3K9 acetylation, which further promotes this transcriptional enhancement. The proteins illustrated in green were specifically observed by ChIP in this study to occupy the *erbB2* promoter.

review board. Gene copy number was analyzed by genomic PCR spanning intron-exon junctions. β-Globin gene was used as internal control. Please see Table S2 for the sequence of primers used.

Cell Lines, Plasmids, and Treatments

B5/589, MCF-7, and BT-20 (ErbB2-low expressing), ZR-75-1 and MDA-MB-175 VII (ErbB2-overexpressing but nonamplified), and BT-474 and SkBr3 (ErbB2-overexpressing and amplified) breast cancer cells were used. Specific gene silencing was achieved by using either pTripZ (Open Biosystems) or Tet-pLKO-puro (Addgene #21915) vectors. Human Wdr5 ORF was PCR amplified and cloned into pCDNA3 using EcoRI and XhoI enzymes. Trastuzumab, obtained from Genentech (#NDC 50242-056-56), was provided by Dr. George Raptis (Mount Sinai Medical Center).

Real-Time Quantitative PCR and Western Blotting

RNA extraction and quantitative real-time PCR was performed as described previously (Mungamuri et al., 2012). Western blotting was performed as described previously (Mungamuri et al., 2006), with the exception that Alexa Fluor secondary antibodies were used. All blots were developed using the Odyssey fluorescence image scanner, and the band intensities were quantified using LI-COR software. Please see Table S2 for the sequences of primers used.

Quantitative ChIP PCR Assay

ChIP assay was performed as described previously (Mungamuri et al., 2012). Quantitative real-time PCR was performed on the eluted DNA to identify the amount of target sequence. Please see Table S2 for the sequences of primers used.

Generation of Stable Cell Lines

B5/589, ZR-75-1, and SkBr3 cells were infected either with pTripZ:sh-GFP, pTripZ:sh-Wdr5, pTripZ:sh-Gcn5, Tet-pLKO-shGFP, Tet-pLKO-shAsh2L, or with Tet-pLKO-shPCAF lentivirus and selected for puromycin (2 $\mu g\ ml^{-1}$) resistance. Stable puromycin-resistant clones were pooled to generate doxycycline-inducible shRNA cell lines. The shRNA expression was induced with 1 $\mu g\ ml^{-1}$ doxycycline for 48 hr.

MTT Assay

The MTT assay, which measures the conversion of the MTT tetrazolium salt into blue formazan crystals by living cells, was performed as described previously (Mungamuri et al., 2006).

Anchorage-Independent Growth by Colony Formation in Soft Agar

SkBr3 and ZR-75-1 cells stably expressing inducible shWdr5 were plated for soft agar assay. Base agar was prepared by mixing 0.8 ml of 3% agar to 4.2 ml of complete medium (final agar concentration 0.6%) and 1 ml was added to each 35 mm dish in triplicate. After the base agar is set, 3% agar solution was mixed with 2×10^4 cells in medium (final agar concentration 0.34%) and layered on the solidified base agar. For shRNA induction, 1 $\mu g\ ml^{-1}$ doxycycline was added to complete medium and 200 μl of fresh medium with doxycycline was overlaid every alternative day. The plates were incubated at 37°C for up to 3 weeks. Colonies were counted and microphotographed.

For further details, please refer to the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, thirteen figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.09.009.

AUTHOR CONTRIBUTIONS

S.K.M. and S.A.A. planned the project. S.K.M. conducted all the experiments, with some assistance from W.M. and L.G. S.A.A. supervised the study. E.B. provided advice for this study. S.K.M. and S.A.A. wrote the manuscript.

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