

SHORT COMMUNICATION

A novel *HER2* gene body enhancer contributes to *HER2* expressionQ Liu¹, MV Kulak², N Borchering³, PK Maina¹, W Zhang³, RJ Weigel^{2,4} and HH Qi¹

The transcriptional regulation of the human epidermal growth factor receptor-2 (*HER2*) contributes to an enhanced *HER2* expression in *HER2*-positive breast cancers with *HER2* gene amplification and *HER2*-low or *HER2*-negative breast cancers following radiotherapy or endocrine therapy, and this drives tumorigenesis and the resistance to therapy. Epigenetic mechanisms are critical for transcription regulation, however, such mechanisms in the transcription regulation of *HER2* are limited to the involvement of trimethylated histone 3 lysine 4 (H3K4me3) and acetylated histone 3 lysine 9 (H3K9ac) at the *HER2* promoter region. Here, we report the identification of a novel enhancer in the *HER2* 3' gene body, which we have termed *HER2* gene body enhancer (HGE). The HGE starts from the 3' end of intron 19 and extends into intron 22, possesses enhancer histone modification marks in specific cells and enhances the transcriptional activity of the *HER2* promoters. We also found that TFAP2C, a known regulator of *HER2*, binds to HGE and is required for its enhancer function and that DNA methylation in the HGE region inhibits the histone modifications characterizing enhancer and is inversely correlated with *HER2* expression in breast cancer samples. The identification of this novel enhancer sheds a light on the roles of epigenetic mechanisms in *HER2* transcription, in both *HER2*-positive breast cancer samples and individuals with *HER2*-low or *HER2*-negative breast cancers undergoing radiotherapy or endocrine therapy.

Oncogene (2018) 37, 687–694; doi:10.1038/onc.2017.382; published online 16 October 2017

INTRODUCTION

Human epidermal growth factor receptor-2 (*HER2*)/Erb-B2 receptor tyrosine kinase 2 is a member of the erbB-like oncogene family, its overexpression occurs in approximately 20–30% of breast cancers¹ and is strongly associated with poor prognosis.² *HER2* has roles in the development of *HER2*-positive breast cancers^{3,4} and resistance to therapy in *HER2*-low or *HER2*-negative breast cancers, in which *HER2* is transcriptionally upregulated by radiotherapy or by endocrine therapy.^{5–7} *HER2* gene amplification is a major mechanism for *HER2* overexpression, however, higher transcription rate of *HER2* per gene copy was also observed in *HER2*-amplified breast cancer cells,^{8–10} that is, *HER2* mRNA levels are 4- to 8-fold and 64- to 128-fold higher in *HER2*-overexpressing and *HER2*-amplified breast cancer cells, respectively, than would be expected from *HER2* gene copy numbers.⁸ A run-on assay showed SKBR3 cells displayed about two fold *HER2* transcription rate higher than in BT474 cells.¹¹ Transcription factors such as TFAP2,^{12,13} Sp1,¹⁴ PBP,¹⁵ YY1,¹⁶ ETS,¹⁷ YB-1¹⁸ and EGR2¹⁹ have been shown to positively regulate *HER2*, whereas MYB,²⁰ FOXP3,²¹ GATA4,²² PEA3,²³ MBP-1,²⁴ NOTCH and RBP-Jk²⁵ have negative effects. Most of these studies focused on the regulation of the originally characterized *HER2* promoter (promoter 2),^{14,26,27} which has the dominant role in the overexpression of *HER2* in breast cancers²⁸ despite of the identification of an alternative promoter (promoter 1)²⁹ (Figure 1a). Moreover, intron 1 enhancer when bound by PAX2 mediates transcriptional repression of *HER2* by activated ER.⁵ Although these transcriptional mechanisms can

explain *HER2* regulation in part, the molecular basis of the increase in *HER2* transcription in certain cancers remains unexplained.

Chromatin modifications can greatly influence transcriptional regulation and contribute to cancer development.³⁰ H3K4me3 and H3K9ac, two histone marks typically associated with gene activation, were reported to be critical for inducing *HER2* transcription through promoter 2.¹⁰ WDR5, a key component of the H3K4me3 methyltransferase complex, is essentially involved in this process.¹⁰ However, these mechanisms are common to general transcriptional activation. Thus, additional mechanisms may exist and specifically contribute to *HER2* overexpression. We discovered a novel enhancer *HER2* gene body enhancer (HGE) in the 3' gene body of *HER2*.

The HGE activates promoters 1 and 2 *in trans.*, and hence the TFAP2C-mediated transcriptional induction of *HER2* expression. This novel regulatory mechanism of *HER2* transcription contributes to the understanding of increased expression of *HER2*.

RESULTS AND DISCUSSION

Identification of a novel enhancer in *HER2* locus

Enhancer interacts with promoter to recruit RNA polymerase II and regulate transcription.³¹ Chromatin signatures such as DNase I hypersensitivity sites³² and histone modifications, that are, H3K4me1 and H3K27ac can be used to predict putative enhancers.^{33–35} We took advantage of data from the Encyclopedia of DNA Elements (ENCODE) Consortium³⁶ to search for novel regulatory element(s) that may contribute to the increase of *HER2*

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Received 29 March 2017; revised 9 August 2017; accepted 8 September 2017; published online 16 October 2017

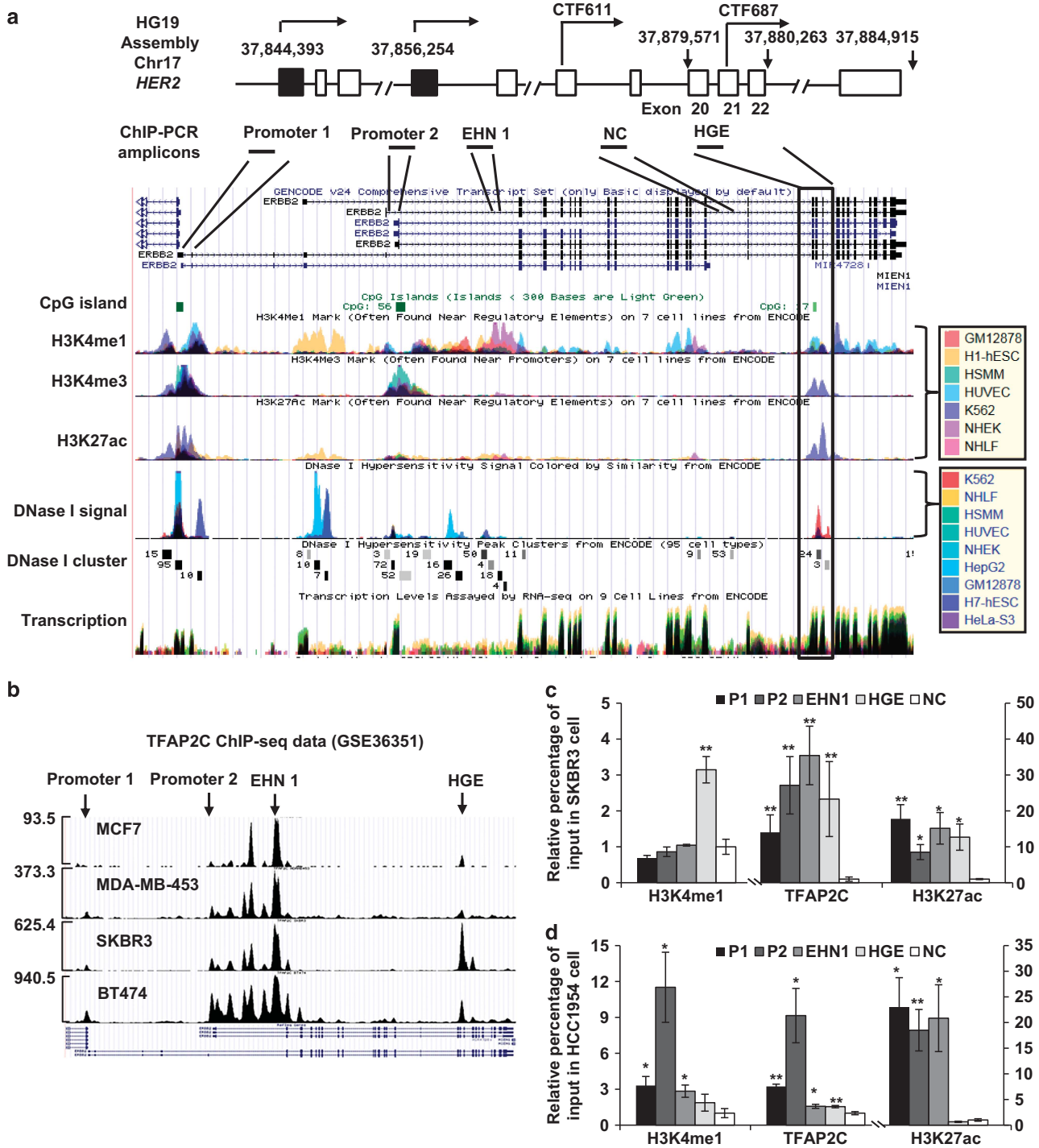


Figure 1. Epigenetic demarcation of the HGE. **(a)** Top: schematic illustration of the locations of the *HER2* promoters (1 and 2), two C-terminal transcripts (CTF611 and CTF687), and the HGE region, as well as the exon composition of *HER2*, based on UCSC gene annotation (GRCh37/hg19). Bottom: enrichment of H3K4me1, H3K4me, H3K27ac and DNase I hypersensitivity signals, retrieved using the ENCODE Regulation Super-track Settings. Colors representing different cell types are shown in the legend. Chromatin immunoprecipitation (ChIP)-PCR amplicons are shown. NC: sequences to which nonspecific controls were generated. **(b)** Profile of TFAP2C binding to the *HER2* gene in the indicated cell lines, as retrieved from ChIP-seq data (GSE36351). The maximum reads on the y axis represent normalized coverage (reads per million mapped). **(c, d)** TFAP2C binding and H3K4me1 and H3K27ac modification, in SKBR3 **(c)** and HCC1954 **(d)** cells, as assessed by ChIP. Enrichment was interpreted as a percentage of input. Fold change over input normalized to NC is shown. P1, *HER2* promoter 1; P2, *HER2* promoter 2; EHN1, intron 1 enhancer. Mean \pm s.d. was determined for three independent experiments, and the Student's *t*-test was used to calculate the significance. **P* < 0.05, ***P* < 0.001.

transcription. With the ENCODE Regulation Super-track Settings, DNase I hypersensitivity sites, H3K4me1 and H3K27ac were found in the previously identified intron 1 enhancer⁵ (using the annotation of NM_001005862); 5-kb upstream of promoter 2; and in a previously undiscovered region within the 3' gene body (Figure 1a). The 3' gene body region starts from intron 19 and ends within intron 22 (based on NM_001289936) (Figure 1a) and, in addition to the above-described features, it contains binding sites for many transcription factors (including POLII, TEAD4, c-MYC and PHF8) identified in K562 cells (Supplementary Figure 1). We named this region as the HGE.

TFAP2C is known to positively regulate *HER2* expression as it is required for the *HER2* expression in BT474 cells;³⁷ binds to and regulates *HER2* promoter 2;^{12,38,39} mediates the repression of *HER2* by estrogen;³⁸ its expression is positively correlated with *HER2* expression in primary breast cancers.³⁷ We analyzed the TFAP2C occupancy data from four cell lines (GSE36351)⁴⁰ and revealed that TFAP2C is enriched at both promoter 2 and intron 1 enhancer in all cell lines, with stronger enrichments in *HER2*-amplified SKBR3 and BT474 lines than in *HER2*-low MCF7 cells (Figure 1b). Importantly, TFAP2C also binds to the HGE in both SKBR3 and BT474 cells, with the stronger occupancy in SKBR3 cells than in BT474 cells (Figure 1b), which supports HGE as a candidate enhancer. Chromatin immunoprecipitation of TFAP2C, H3K4me1 and H3K27ac were performed in SKBR3 cells and TFAP2C occupancies were confirmed at promoters 1 and 2, the intron 1 enhancer and HGE (Figure 1c). H3K27ac is enriched at all four regions, whereas H3K4me1 enrichment was only obvious at HGE (Figure 1c). Chromatin immunoprecipitation experiments in another *HER2*-amplified breast cancer cell line, HCC1954 (Figure 1d), revealed that TFAP2C was significantly enriched at all four regions, however, its enrichments at both enhancers were dramatically lower than at promoter 2. H3K27ac was significantly enriched at both promoters and intron 1 enhancer, whereas, H3K4me1 was more enriched at promoter 2 compared with a slight increase at promoter 1 and HGE (Figure 1d). These data support the enhancer feature of the HGE and its cell type dependency. Importantly, a chromosome 17-wide binding data of ERR α and PGC-1 β in SKBR3 cells showed binding of ERR α to HGE in addition to intron 1 enhancer and both promoters,⁴¹ indicating that the HGE can recruit additional transcription factors.

The HGE enhances the transcriptional activity of *HER2* promoters. We tested the ability of the HGE to regulate the transcriptional activities of the *HER2* promoters by luciferase reporter assay in 293T, SKBR3 and BT474 cells (Figure 2a). Both promoters (placed upstream of the *Luciferase* gene), but not the HGE (placed downstream of *Luciferase* gene), had basal activities in all cases (Figure 2a). Notably, in all three cell lines, the transcriptional activity associated with promoter 2 was stronger than that associated with promoter 1 (Figure 2a), consistent with a previous study.²⁸ Importantly, when HGE was placed at the enhancer position in pGL3-basic vector, that is, 5' to 3' downstream of the *Luciferase* gene, it enhanced the transcriptional activities of both *HER2* promoters in all three cell lines (Figure 2a). As H3K4me3 presents at HGE in K562 cells and *HER2* C-terminal fragment (CTF) 687 uses exon 21 to initiate its transcription⁴² (Figure 1a), we engineered HGE upstream of the *Luciferase* gene. Only a minor transcriptional activity was detected in 293T cells (Figure 2b), suggesting that the transcriptional initiation capacity of HGE is much weaker compared with that of *HER2* promoters. Interestingly, HGE, when placed immediately upstream of the *Luciferase* gene significantly interrupted the transcriptional activities of both promoters (Figure 2b). Meanwhile, the HGE maintained its enhancer function for both *HER2* promoters when it was inversely inserted into the enhancer position, that is, 3' to 5' downstream of the *Luciferase* gene (Figure 2c). Moreover, when compared with

Intron 1 enhancer, the HGE possesses similar enhancer function for promoter 1 but slightly weaker for promoter 2 (Figure 2c). These data support that the HGE has enhancer function comparable with the intron 1 enhancer.

TFAP2C regulates the enhancer function of HGE

To determine the minimal enhancer element of HGE, we generated a series of deletions including the deletion of exon 20 and intron 20 (T20), the deletion of exon 21 and intron 21 (T21) and the deletion of exon 22 and intron 22 (T22) (Figure 3a, left panel). The transcriptional activities of these constructs were analyzed using the luciferase assay in 293T and SKBR3 cells. The T21 deletion abolished the enhancement of transcriptional activity from promoter 2 in both cell lines, whereas the T20 deletion had no significant effect in either case, and the T22 deletion increased the transcriptional activity of promoter 2 (Figure 3a). These data suggested that exon 21 and intron 21 contain sequences involved in transcriptional activation, and exon 22 and intron 22 contain sequences involved in transcriptional repression. We next searched for the TFAP2C consensus sequence (GCCTGAGGG)⁴³ and identified three closest potential TFAP2C-binding sites (GCCCCAGAG, GCCCTAGGG, GCCCAGGGC) (Figure 3b) located within intron 21. An electrophoretic mobility shift assay using *in vitro* translated TFAP2C and three oligonucleotide probes corresponding to the three potential TFAP2C-binding sites showed that TFAP2C binds to all the three probes (Figure 3b). The specificity of TFAP2C binding was further confirmed by competition of non-labeled oligos and the supershifts when cultured with a specific TFAP2C antibody (Figure 3b). TFAP2C silencing attenuated luciferase activity of the HGE enhancer in both 293T and SKBR3 cells (Figure 3c) and downregulated *HER2* protein levels in SKBR3 cells. (Figure 3d). These data support our hypothesis that TFAP2C has an important role in regulating the enhancer function of the HGE.

We next carried out genomic editing using CRISPR-Cas9 system to determine the role of the TFAP2C-binding sites at the HGE in the regulation of *HER2* expression. Six single-guide RNAs (gRNA) were designed to mutate or truncate TFAP2C-binding sites (Figure 3b). SKBR3 stable cell lines with the single or combined gRNA(s) were attempted to be established, however, only one stable cell line with gRNA6 was achieved. It is likely that the cell viability of SKBR3 cells depends on *HER2* as knockdown *HER2* in SKBR3 cells results in growth arrest and apoptosis.⁴⁴ *HER2* protein in this cell line was dramatically downregulated while cleaved PARP was induced (Figure 3e). The genotyping of SKBR3-gRNA6 showed heterogenous genomic compositions, that is, normal HGE region and extended mutations from the gRNA6-targeting site (Supplementary Figure 2). This data suggest that CRISPR-Cas9 system with these gRNAs introduced additional mutations in *HER2* gene and may interfere with *HER2* mRNA splicing, resulting in decreased *HER2* expression and apoptosis of SKBR3 cells. In fact, a recent study show that genomic editing of *HER2* gene using CRISPR-Cas9 system produced short truncated *HER2* caused by alternative splicing of *HER2* gene and inhibits cell proliferation in both SKBR3 and BT474 cells,⁴⁵ consistent with a recent observation of off-target mutations introduced by CRISPR-Cas9 system.⁴⁶

Considering the *HER2*-dependent cell viability of SKBR3 cells, the six gRNAs were transiently transfected to SKBR3 cells for 48 h and downregulation of *HER2*, phospho-AKT levels at various extents was found without inducing obvious apoptosis (Figure 3f). These data suggest that the gRNAs targeting intron 21 containing three TFAP2C-binding sites and the junction of exon 21 and intron 21 interfere with *HER2* expression. We also examined the mRNA levels of *TFAP2C* and *HER2* from 12 unidentifiable *HER2*-positive breast cancer samples and found positive correlation ($r^2=0.6073$) between them (Figure 3g).

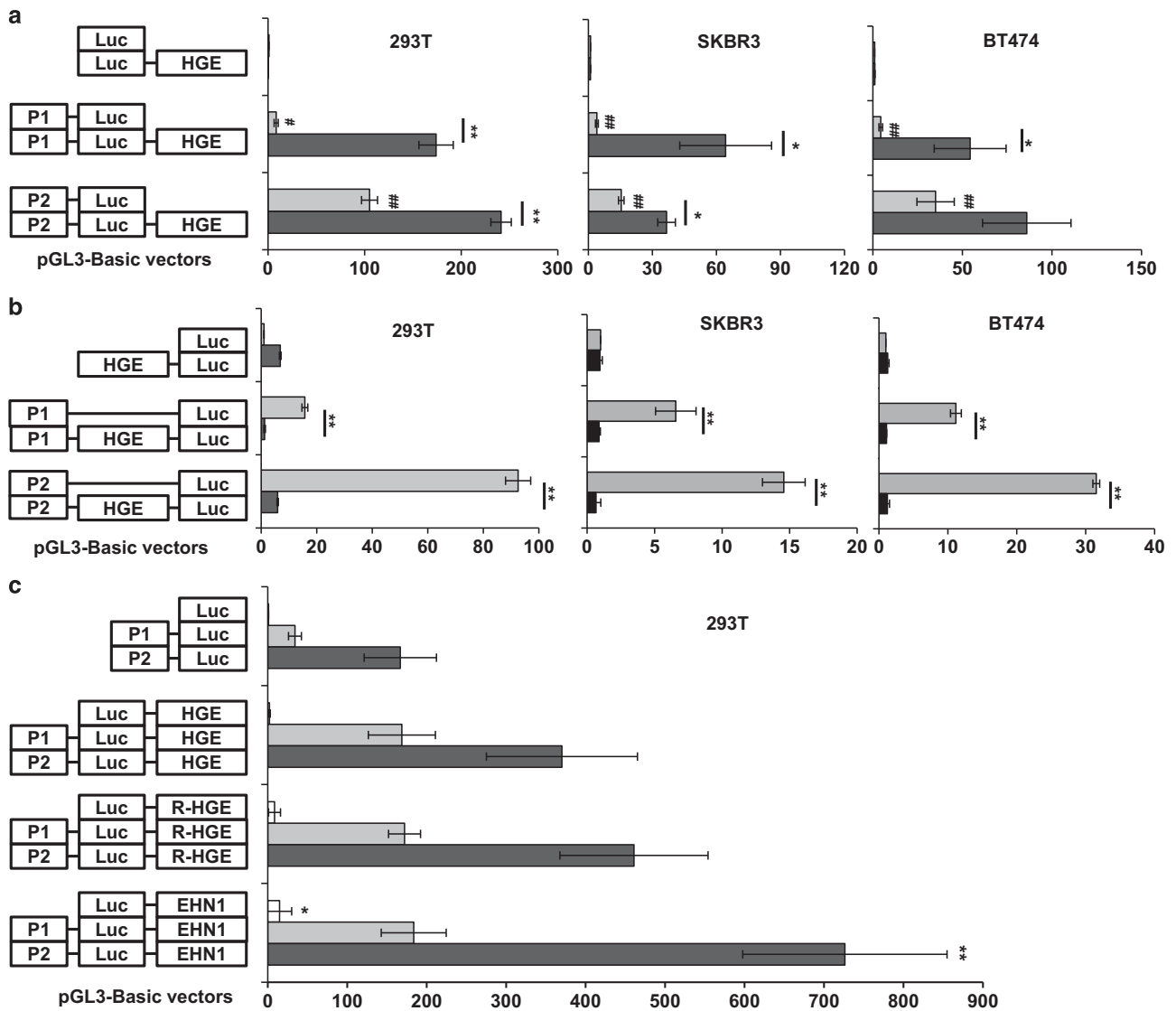


Figure 2. The HGE enhances transcriptional activity of *HER2* promoters. (a) Relative luciferase activities of reporter constructs illustrated at left, normalized to expression of *Renilla* luciferase from co-transfected pRL-TK-Rluc plasmid. (a) Relative luciferase activity elicited by *HER2* promoter 1 (P1), *HER2* promoter 2 (P2) and *Luciferase* (Luc), in 293T, SKBR3 and BT474 cells. # indicates the significance between each group versus pGL3-basic vector. * $P < 0.05$, ** $P < 0.001$. # indicates the significance between each group versus pGL3-basic vector. # $P < 0.05$, ## $P < 0.001$. (c) Comparison of the enhancement activities of HGE, reverse HGE and intron 1 enhancer by luciferase assay. R-HGE, reverse HGE; EHN1, intron 1 enhancer. Student's *t*-test was used to calculate the significance between constructs with R-HGE or EHN1 versus HGE with each promoter separately. No significance was observed between R-HGE and HGE, whereas EHN1 significantly enhance transcriptional activities of the basic vector and *HER2* promoter 2 compare with HGE and R-HGE.

DNA methylation within the HGE inhibits the enhancer histone modifications and is inversely correlated with *HER2* gene expression in breast cancer samples

The cell-type-dependent enrichments of TFAP2C, H3K4me1 and H3K27ac in the HGE region (Figures 1c and d) prompted us to investigate the underlying mechanism. DNA methylation is known to prevent TFAP2C from accessing the target promoter,⁴⁷ and DNA methylation and certain histone modifications such as H3K4me3 are mutually exclusive.⁴⁸ Thus, we hypothesized that DNA methylation within the HGE affects TFAP2C binding and enrichment of the enhancer histone modifications. Analysis of both Methyl 450 K bead array data (ENCODE/HAIB) and Reduced Representation Bisulfite Seq data (ENCODE/HudsonAlpha)³⁶

showed that DNA hypomethylation in the HGE region is coincident with enrichments of transcription factors and enhancer histone modifications in K562 cells (Supplementary Figure 1). We performed a bisulfite sequencing assay to determine the DNA methylation status of 28 CpG sites within the HGE. The HGE is extensively DNA-methylated in MCF7, BT474, HCC1954, MDA-MB-231, MCF10A and ZR-75-1 cells. It is less methylated in K562 and hypomethylated in SKBR3 cells (Figure 4a). These data support our hypothesis that DNA methylation status is critical for the enrichments of TFAP2C, H3K4me1 and H3K27ac in the HGE region. The minor enrichments of TFAP2C at HGE in both BT474 and HCC1954 cells (Figures 1b and d) likely reflect the existence of minor cell populations possessing hypomethylated HGE or the cells toward complete establishment of DNA methylation during

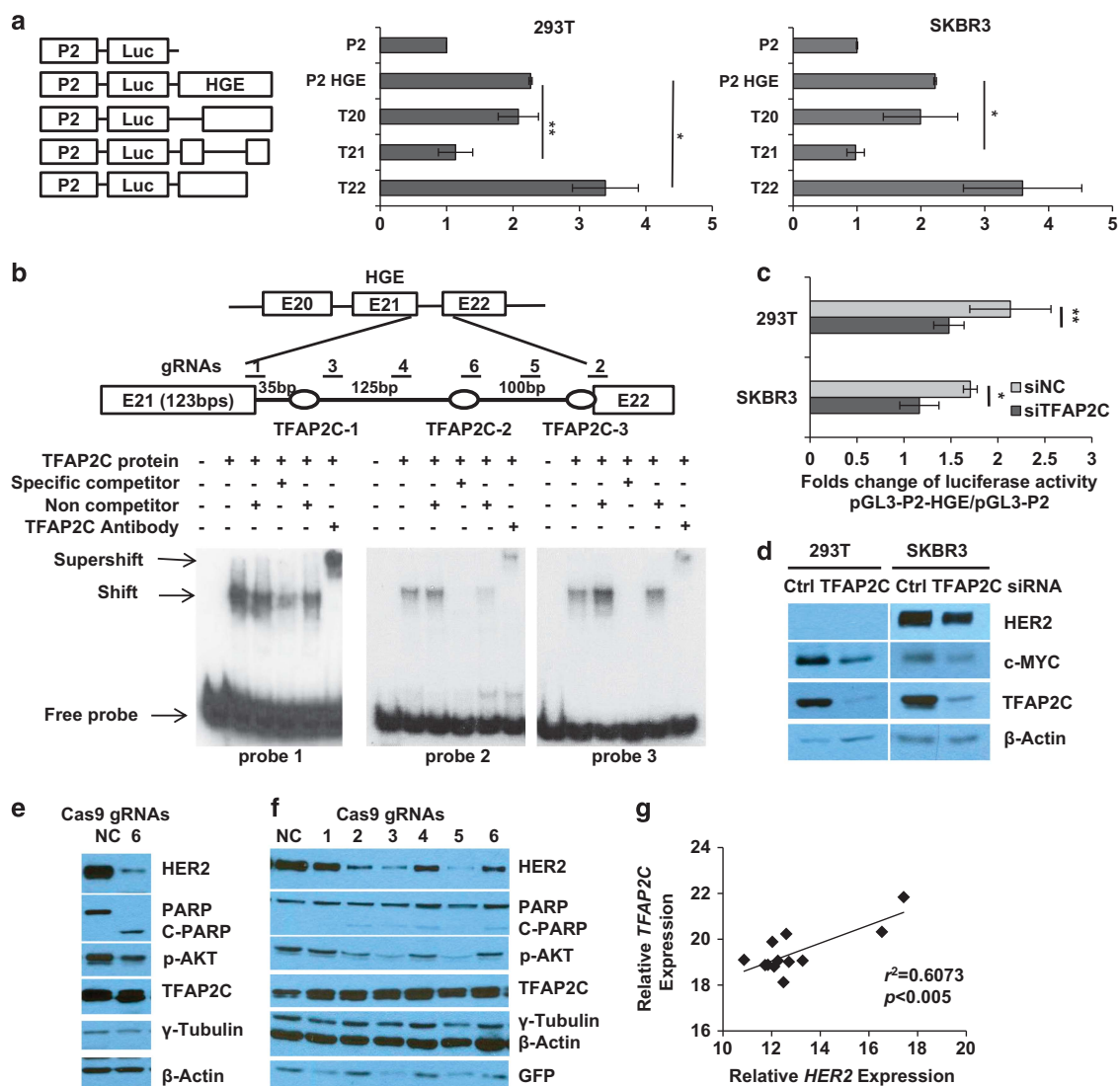


Figure 3. TFAP2C positively regulates enhancer activity of the HGE. **(a)** Relative luciferase activities of reporter constructs bearing deletions in HGE region shown at left (T20: deletion of exon 20 and intron 20 in the pGL3-P2-HGE vector) in 293T and SKBR3 cells. Relative luciferase activities of the indicated constructs normalized to *Renilla* luciferase expression in cells co-transfected with pRL-TK-Rluc. **(b)** Schematic illustration of positions of three potential TFAP2C-binding sites corresponding to three probes for electrophoretic mobility shift assay (EMSA). Unlabeled 25-bp oligonucleotides or nonspecific competitor were added as indicated. + and - indicate the presence or absence of reaction components. Shifts, supershifts and free probes are indicated with arrows. **(c)** Fold change of relative luciferase activity of pGL3-P2-HGE normalized to that of pGL3-P2 vectors in 293T or SKBR3 cells transiently transfected with control (Ctrl) or TFAP2C siRNAs. **(d)** Expression of the indicated proteins as assessed by western blotting. Mean \pm s.d. in **a** and **c** were obtained from three independent experiments. Student's *t*-test was used to calculate the significance. * $P < 0.05$, ** $P < 0.001$. **(e, f)** Six gRNAs were designed to introduce mutations in intron 21 as indicated in **b**. Virus infected **(e)** and transient transfection for 48 h **(f)** of CRISPR-Cas9-GFP vector with corresponding gRNAs in SKBR3 cells were assessed by western blotting. **(g)** RT-PCR used to determine a correlation between *HER2* expression and expression of *TFAP2C* in panel of primary breast cancer samples ($n = 12$). The correlation (r^2) and significance (P -value) are shown.

cell cycle. We next performed *in vitro* methylation of the pGL3-promoter constructs with and without HGE, using the CpG methyltransferase *M.SssI*^{49,50} and found a strong decrease of enhanced luciferase activity of the methylated pGL3-promoters vector with HGE compared with that without HGE (Supplementary Figure 3).

Furthermore, we performed CRISPR-dCas9-guided specific DNA methylation⁵¹ at the HGE region in SKBR3 cells. The six gRNAs (Figure 3b) were inserted into pdCas9-DNMT3A-EGFP vector and again, stable cell lines using SKBR3 cells were failed to establish. However, we were able to collect cells from

gRNA 1 and 6 during the attempt and found that the cells undergo apoptosis (Figure 4b, left panel). Importantly, the HGE region became partially methylated in SKBR3 cells that transfected with pooled gRNAs, validating the target-specific DNA methylation by the dCas9-DNMT3A system and indicating that the apoptosis might be caused at least partially by loss of *HER2* (Figure 4c). The dCas9-DNMT3A-gRNA 1 and 6 transiently transfected into SKBR3 cells for 48 h markedly downregulated *HER2* expression, phospho-AKT, but not that of *TFAP2C* and no apparent apoptosis was observed (Figure 4b, right panel). These data suggest that the DNA methylation at the

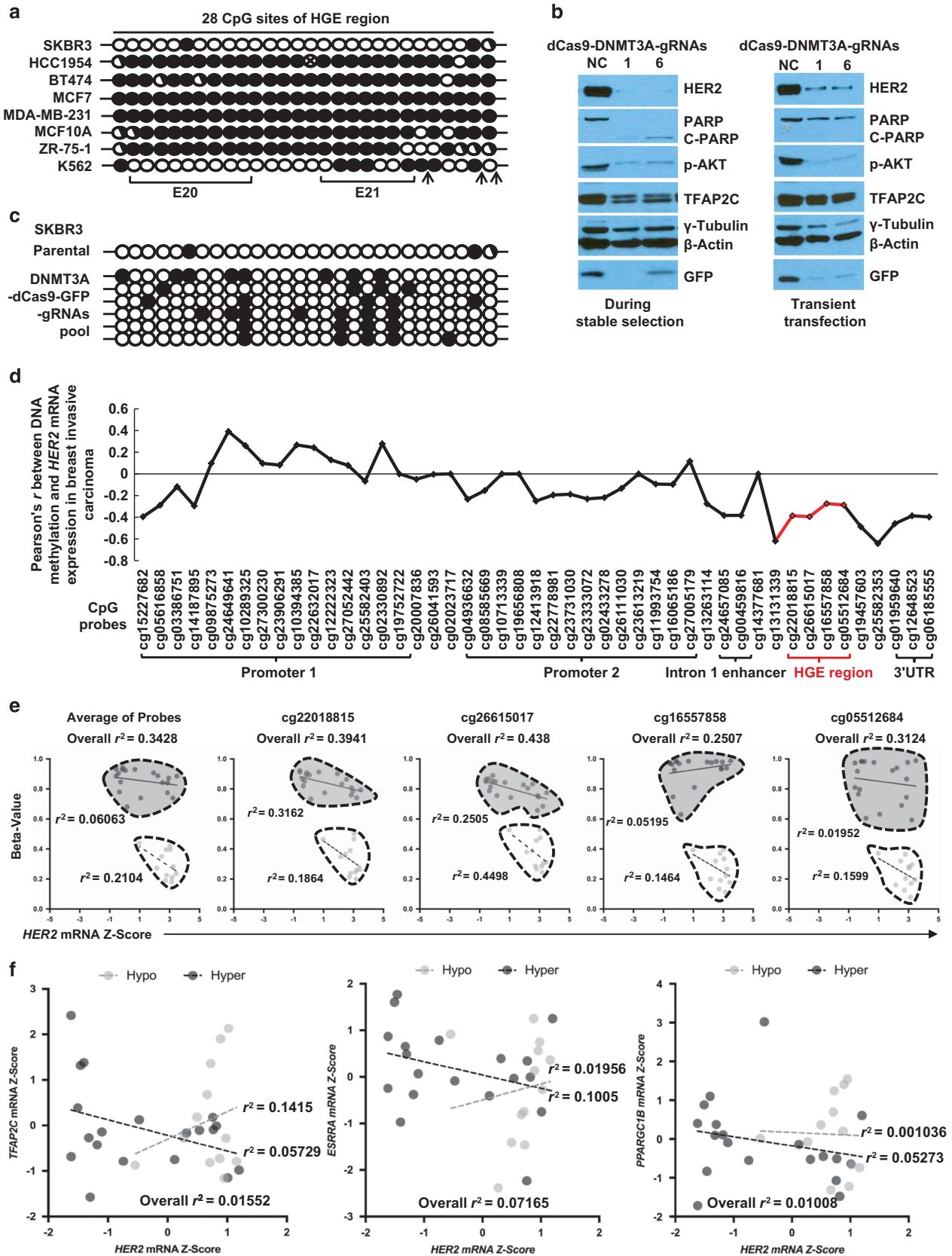


Figure 4. DNA methylation determines the enhancer function of HGE and is inversely correlated with *HER2* gene expression in breast cancers. **(a)** DNA methylation of the 28 CpG dinucleotides in the HGE region in multiple cell lines, assessed using the bisulfite sequencing. Each circle represents a CpG dinucleotide: open circle, unmethylated site; closed circle, methylated site; half-closed circle, semi-methylated site; circle with an x, mutated site. Exons are marked, and TFAP2C consensus nucleotides are indicated by arrows. **(b)** Indicated proteins from SKBR3 cells either transfected with CRISPR-dCas9-DNMT3A-EGFP carrying gRNA 1 or 6 and undergoing stable selection (left) or transiently transfected with the same plasmids for 48 h (right) were assessed by western blotting. **(c)** DNA methylation status of the HGE region in SKBR3 during the stable selection as assessed by bisulfite sequencing. **(d)** Correlation (Pearson's *r*) of all 47 DNA methylation sites around *HER2* gene including HGE regions (red color) with *HER2* expression in 839 breast invasive carcinoma samples was analyzed using MethHC.⁵³ **(e)** The inverse correlation of average and each individual CpG sites located in HGE with *HER2* expression in *HER2*-positive breast cancer samples grouped in hypomethylation (light) and hypermethylation (dark) is shown. Pearson *r*² values of the correlations are displayed in each subgroup of samples. **(f)** Correlation between *TFAP2C*, *ESRRA*, *PPARGC1B* and *HER2* mRNA level in *HER2*-positive breast cancer samples sub grouped by DNA methylation status of HGE region were plotted. Light and dark dots are designated to hypomethylation and hypermethylation in the HGE region, respectively.

HGE region is important for repression of *HER2* expression in SKBR3 cells.

DNA methylation is in general strongly correlated with *HER2* expression ($r^2 = 0.5055868$) in breast cancer.⁵² Using the methylation database MethHC,⁵³ we analyzed the correlation of the DNA methylation status of 47 CpG probes and *HER2* expression in 839 breast invasive carcinoma samples cataloged in The Cancer Genome Atlas. The general inverse correlation of DNA methylation in *HER2* gene body and *HER2* mRNA is stronger ($r = -0.48$) compared with that of the promoters ($r = -0.22$ and -0.19 for promoters 1 and 2, respectively) (Supplementary Figure 4). The correlation of the DNA methylation status of all 47 CpG probes with *HER2* mRNA expression shows strong inverse correlation (*r* values ranging from -0.291 to -0.408) between the DNA methylation of the four HGE CpG sites and *HER2* mRNA expression (Figure 4d). Further analysis in *HER2*-positive breast cancers revealed that the DNA hypomethylation of all four HGE CpG sites is inversely correlated with *HER2* mRNA (Figure 4e). In the samples with hypomethylated HGE region, *TFAP2C* mRNA is positively associated with *HER2* mRNA (Figure 4f). As discussed earlier that ERRA also binds to HGE region and regulates *HER2* expression in SKBR3 cells,⁴¹ we also found a positive, but weaker association between the mRNA of the coding gene *ESRRA* and *HER2* expression. In contrast, the expression of *PPARGC1B* does not show any association. These data suggest that the hypomethylation of the HGE region in breast cancers contributes to *HER2* expression by gaining the accessibility of transcription factors such as *TFAP2C* and ERRA.

In sum, this study unveiled a novel regulatory mechanism by a 3' gene body enhancer contributing to the transcriptional regulation of *HER2*. Further studies are sought to determine the role of this enhancer in the transcriptional upregulation of *HER2* in *HER2*-low or *HER2*-negative breast cancers that undergo radiotherapy or endocrine therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dr Brad Amendt and his lab for helpful discussions, and the ENCODE Consortium and the ENCODE production laboratories for generating the relevant data sets. The results of the bioinformatical analysis are based, in whole or part, upon data generated by The Cancer Genome Atlas Research Network (<http://cancergenome.nih.gov/>). The K562 cell line was a kind gift from Dr Fenghuang Zhan. We also thank Drs Christine Blaumueller and Marie Gaine for editorial consultation. This work was supported by to HHQ start-up funds from the Department of Anatomy and Cell Biology, the Carver College of Medicine, University of Iowa; Carver Trust Young Investigator Award (01-224 to HHQ) from the Roy J Carver Charitable Trust; a Breast Cancer Research Award (to HHQ) by the Holden Comprehensive Cancer Center at University of Iowa; The NIH grant (P30 CA086862) to the Genomics and Flow Cytometry core facilities at the University of Iowa. NIH grants R01CA183702 (PI: RJW) and by a generous gift from the Kristen Olewine Milke Breast Cancer Research Fund

(PI: RJW). NB was supported by NIH MD/PhD fellowship (F30 CA206255); WZ was supported by NIH grants CA200673, and CA203834, the V Scholar award, a Breast Cancer Research Award and an Oberley Award (National Cancer Institute Award P30 CA086862) from Holden Comprehensive Cancer Center at the University of Iowa.

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