ABSTRACT

Understanding factors required for DNA replication will enrich our knowledge of this important process and potentially identify vulnerabilities that can be exploited in cancer therapy. We applied an assay that measures the stability of maintenance of an episomal plasmid in human tissue culture cells to screen for new DNA replication factors. We identify an important role for DDX5 in G1–S-phase progression where it directly regulates DNA replication factor expression by promoting the recruitment of RNA polymerase II to E2F-regulated gene promoters. We find that the DDX5 locus is frequently amplified in breast cancer and that breast cancer–derived cells with amplification of DDX5 are much more sensitive to its depletion than breast cancer cells and a breast epithelial cell line that lacks DDX5 amplification. Our results show a novel role for DDX5 in cancer cell proliferation and suggest DDX5 as a therapeutic target in breast cancer treatment.

SIGNIFICANCE: DDX5 is required for cell proliferation by controlling the transcription of genes expressing DNA replication proteins in cancer cells in which the DDX5 locus is amplified, and this has uncovered a dependence on DDX5 for cell proliferation. Given the high frequency of DDX5 amplification in breast cancer, our results highlight DDX5 as a promising candidate for targeted therapy of breast tumors with DDX5 amplification, and indeed we show that DDX5 inhibition sensitizes a subset of breast cancer cells to trastuzumab. Cancer Discov; 2(9): 1–14. ©2012 AACR.
INTRODUCTION

Defects in the control of cell proliferation are a hallmark of cancer, and DNA replication is a key process for cell proliferation. Understanding how DNA replication is regulated in human cells can provide insight into cancer development and may reveal vulnerabilities that can be exploited therapeutically. Indeed, a number of agents currently used in cancer treatment are known to target DNA synthesis. We applied an assay that is loosely based upon the minichromosome maintenance screen conducted in budding yeast (1) in which we measured the stability of maintenance of an episomal plasmid in human tissue culture cells to screen for new factors required for DNA replication. The plasmid encodes both the \textit{EBNA1} gene and OriP, a \textit{cis}-acting element derived from the Epstein Barr virus (EBV) genome that functions as an origin of DNA replication in plasmids and enables them to tether to chromosomes during mitosis, thereby allowing the plasmids to replicate and segregate to daughter cells without the need to integrate into host chromosomes (2). To be maintained in cells, OriP plasmids require both the \textit{EBNA1} protein, which is encoded by a gene also derived from the EBV genome, and host cell DNA replication factors (3, 4). Importantly, OriP plasmids replicate once per S-phase and are thus licensed to replicate similar to human chromosomes (5). Our rationale for using this episomal plasmid for the screen is that stable maintenance of a plasmid whose duplication in cells is dependent upon a single origin of replication should be more sensitive to reduced expression of DNA replication factors than chromosomes that encode many origins of replication. We identified 6 genes that had no appreciated role in DNA replication but whose expression is necessary for stable plasmid maintenance in cells and present evidence that one of these, the DEAD-box protein 5 (DDX5), regulates the expression of DNA replication genes and is required for cancer cell proliferation.

DDX5 is an ATP-dependent RNA helicase that was first identified as a protein that cross-reacted with an antibody against SV40 large T-antigen (6, 7). It exhibits considerable sequence identity in its helicase core with the DEAD-box protein DDX17 and these 2 proteins interact in cells (8). DDX5, and in fewer cases DDX17, can function as transcriptional co-regulators with estrogen receptor-\textit{α}, p53, MyoD, and Runx2 (9). A role for DDX5 and DDX17 in miRNA maturation, ribosome biogenesis, mRNA splicing, and insulator function have also been described (10–16). DDX5 is frequently overexpressed in colon, prostate, and breast cancer (17–20), and RNA interference (RNAi) of both DDX5 and DDX17 together impairs cancer cell proliferation (13, 19). However, the activity of DDX5 in promoting cell proliferation is poorly understood.

We find that in certain cancer cells, DDX5 is required for plasmid stability and cell proliferation where it promotes \textit{G}_{1}–S-phase progression and the expression of essential DNA
suggesting a vulnerability that may be targeted by therapy. Show that breast cancer cells acquire dependence upon DDX5, and is correlated with dependence of breast cancer cells on DDX5 expression varies breast epithelial cell line lacking this amplification. Thus, the its knockdown than breast cancer cell lines and a normal amplification of the DDX5 locus is frequently co-amplified along ERBB2. Surprisingly, breast cancer cell lines with amplification of DDX5 overexpression previously reported to reduced expression of an essential replication factor than containing a single origin of replication would be more sensitive to strong than cell proliferation.

RESULTS

Plasmid Stability Assay to Identify Genes Required for DNA Replication in Human Cells

An assay that measures stability of maintenance of an episomal plasmid in human tissue culture cells was applied to the human colorectal tumor cell line HCT116 to identify new replication genes. DDX5 localizes to the E2F-regulated promoters of genes encoding DNA replication factors and is required for RNA polymerase II loading. Consistent with its positive regulation of genes required for cell division and the high frequency of DDX5 overexpression previously reported in breast cancer, we find that the DDX5 locus is frequently amplified in breast cancer and is often co-amplified along with ERBB2. Surprisingly, breast cancer cell lines with amplification of the DDX5 locus are considerably more sensitive to its knockdown than breast cancer cell lines and a normal breast epithelial cell line lacking this amplification. Thus, the dependence of breast cancer cells on DDX5 expression varies and is correlated with DDX5 gene copy number. These results show that breast cancer cells acquire dependence upon DDX5, suggesting a vulnerability that may be targeted by therapy.

Figure 1. Plasmid maintenance screen. A, schematic of plasmid stability assay used for screen. Puro, puromycin. B, quantitative Western blot analysis of MCM3 and PCNA (loading control) in whole-cell extracts obtained from cells transduced with the indicated shRNAs. V2HS_262054 is an shRNA that targets MCM3 expression. The Luciferase mi203 whole-cell extract is loaded such that there is either equal total protein loaded as the V2HS_262054 whole-cell extract (lane indicated as “1.00” on the blot) or 25% or 10% total protein loaded as the V2HS_262054 whole-cell extract (lanes indicated as “0.25” and “0.10,” respectively). PCNA, proliferating cell nuclear antigen. C, colony formation assay results for triplicate HCT116 cultures transduced with either V2HS_262054, EBNA1 mi1666, or Luciferase mi203 shRNAs that were grown in either growth media + puromycin (proliferation assay) or growth media + puromycin and hygromycin (plasmid stability assay). D, plot of plasmid stability ratios calculated from results shown in (C). E, plasmid stability ratios for shRNAs tested in screen. Blue bars on plot correspond to plasmid stability ratios for HCT116 cultures transduced with shRNAs that target expression of known replication factors.
DDX5 controls cancer cell proliferation. Six of the 55 genes we tested met this criterion (Supplementary Table S1) that included 44 shRNAs targeting the expression of 16 known DNA replication factors. The remaining 186 shRNAs targeted 55 genes of poorly characterized function that are either upregulated in expression during the G1 and S-phases of the cell cycle (26) or encode proteins found to interact with human ORC (S. Prasanth and B. Stillman, unpublished data). Selection of shRNAs tested in the screen was not based upon prior experimental data showing efficacy, and so variable potency of gene knockdown was obtained with these reagents, ranging from poor to strong knockdown of gene expression. Twenty-six shRNAs targeting the expression of 14 known DNA replication factors impaired plasmid stability with ratios of 0.45 or less, consistent with plasmid stability being a useful measure for shRNAs that target plasmid maintenance while having little effect on cell proliferation (Fig. 1D).

We tested the effect of 230 different shRNAs on cell proliferation and plasmid stability (Supplementary Table S1) that included 44 shRNAs targeting the expression of 16 known DNA replication factors. The remaining 186 shRNAs targeted 55 genes of poorly characterized function that are either upregulated in expression during the G1 and S-phases of the cell cycle (26) or encode proteins found to interact with human ORC (S. Prasanth and B. Stillman, unpublished data). Selection of shRNAs tested in the screen was not based upon prior experimental data showing efficacy, and so variable potency of gene knockdown was obtained with these reagents, ranging from poor to strong knockdown of gene expression. Twenty-six shRNAs targeting the expression of 14 known DNA replication factors impaired plasmid stability with ratios of 0.45 or less, consistent with plasmid stability being a sensitized reporter of DNA replication (Fig. 1E). Western blotting revealed that shRNAs yielding plasmid stability ratios greater than 0.45 are less potent at knocking down their gene targets compared with shRNAs that target the same gene but yield plasmid stability ratios of less than 0.45 (Supplementary Fig. S1A–S1D).

We established the criteria for consideration as a hit in the screen as any gene in which 2 or more shRNAs targeting its expression yielded plasmid stability ratios of 0.45 or less. Six of the 55 genes we tested met this criterion (Supplementary Table S2). One gene, AND-1, was a gene of unknown function at the time it was tested but has since been shown to have an essential role in DNA replication, where it interacts with MCM10 and is required for loading of DNA polymerase-α onto origins of replication (27). This validated the use of this assay in identifying new DNA replication factors. Other hits from the screen included the genes encoding DEAD-box protein DDX5 (p68), the condensin II subunit NCAP-G2, the PLK1-interacting protein PICH, and 2 proteins of unknown function, C14ORF130 and KDELCL1. For DDX5, AND-1, and NCAP-G2 knockdown correlated well with the observed plasmid stability ratios where the more potent shRNAs targeting the expression of these genes resulted in the greatest impairment of plasmid stability (Supplementary Fig. S2A–S2C).

DDX5 is known to interact with the related DDX17, and in some cases, but not all, they share overlapping activities in cells. shRNAs targeting DDX17 were included in the screen, and despite 10-fold knockdown of DDX17, none of them impaired plasmid stability (Supplementary Fig. S3A and S3B). This was in contrast to DDX5 shRNAs that showed 4-fold knockdown of DDX5 protein level and strongly impaired plasmid stability (Supplementary Fig. S2B). This result suggested that the activity of DDX5 that underlies its role in plasmid stability was separable from its overlapping activities with DDX17 in the cell.

DDX5 is required for both plasmid stability and cancer cell proliferation. Additional shRNAs targeting DDX5 expression were tested to verify the requirement for DDX5 in plasmid stability (Fig. 2A). All the DDX5 shRNAs strongly impaired plasmid stability compared with cells transduced with the FLuciferase shRNA (Fig. 2B). The most potent DDX5 shRNAs, DDX5mi2053, DDX5mi1314, and DDX5mi2008, also strongly impaired HCT116 proliferation (Fig. 2C) showing a requirement for DDX5 in proliferation in this cancer cell line. Interestingly, DDX5 is overexpressed in HCT116 cells compared with non-tumor colon cell lines (19). Despite the sensitivity of HCT116 cells to DDX5 depletion, we noted that not all cell lines required DDX5 to proliferate (see below) and so we investigated why HCT116 cells were dependent upon DDX5.

DDX5 is required for entry into S-phase. We were interested in whether DDX5 contributes toward DNA replication, so the effect of depletion of DDX5 on cell-cycle progression was examined. siRNAs were used to knockdown DDX5 as their effects on cells were more acute. The DDX5si2008 and DDX5si2053 siRNAs were selected, as they were the most potent at depleting DDX5 when embedded within the mir30-based shRNA backbone (Fig. 2A). The EBNA1si1666 siRNA was used as the negative control, because EBNA1 is not an endogenous protein in HCT116 cells and because the EBNA1si1666 shRNA did not impair cell proliferation (Fig. 1C). Within 48 hours after siRNA transfection, DDX5 was knocked down 90% or greater by both DDX5 siRNAs, with the DDX5si2008 siRNA being more potent than the DDX5si2053 siRNA (Fig. 3A). Knockdown of DDX5 resulted in an increased fraction of cells in G1 phase and a reduced fraction of cells in S-phase (Fig. 3B, Supplementary Fig. S4A). Moreover, the efficiency of DNA replication in S-phase cells is reduced upon DDX5 depletion (Supplementary Fig. S4B). Next, we investigated whether DDX5 depletion impaired cell-cycle entry and progression following addition of serum to serum-starved cells. Cells were transfected for 24 hours with the indicated siRNAs and then they were incubated in serum-free media for another 48 hours. Serum was then added to the media and cell-cycle progression monitored using flow cytometry and Western blot analysis. Following serum addition, cells with DDX5 knockdown progressed slower into S-phase than cells transfected with the negative control siRNA (Fig. 3C). DDX5 depletion did not block the ability of cells to re-enter the cell cycle and progress through G2 phase, as RB hyperphosphorylation and expression of G2–S cyclins were unaffected (Fig. 3D). We also analyzed both the expression and loading onto chromatin of different subunits of the CMG complex, which is required for initiation of DNA replication and hence cell-cycle progression into...
S-phase, where it functions to unwind duplex DNA ahead of DNA synthesis (28). The expression of different subunits of the CMG complex, including MCM2, MCM5, and CDC45, were impaired (Fig. 3D). Moreover, the loading of these subunits onto chromatin at time points corresponding to when cells entered and progressed through S-phase was impaired by DDX5 depletion (Fig. 3E). We conclude that DDX5 contributes toward initiation of replication and thus S-phase entry, where it promotes DNA replication pre-initiation complex assembly on chromatin. Because both episomal plasmid stability and cell proliferation were dependent on MCM5 and CDC45 expression (Supplementary Fig. S1D and Supplementary Table S1), the reduced expression of both these proteins in cells with DDX5 knockdown likely contributes toward the requirement of DDX5 for plasmid stability and cell proliferation. Because DDX5 depletion resulted in reduced abundance of multiple different CMG proteins in whole-cell extracts, this suggests that DDX5 indirectly contributes toward DNA replication by regulating the expression of DNA replication genes.

We were interested in conducting RNAi rescue experiments with wild-type and various mutant DDX5 transgenes in HCT116 cells to identify key amino acids in DDX5 that are required for its activity in cell-cycle progression. While we could detect overexpression of the transcript encoding the RNAi-resistant DDX5 transgene (Supplementary Fig. S5A and S5B), we were unable to obtain a sufficient level of RNAi-resistant DDX5 protein expression in either HCT116 cells or other cell lines for RNAi rescue experiments (Supplementary Fig. S5C). These results suggest that expression of DDX5 is tightly regulated in cells, and we are currently investigating this regulation to develop a method to achieve sufficient RNAi-resistant DDX5 transgene expression for these studies.

Figure 2. DDX5 is required for plasmid stability and cell proliferation. A, quantitative Western blot analysis of DDX5 and β-actin (loading control) in whole-cell extracts obtained from cells transduced with the indicated shRNAs (V2HS_26045 and V2HS_24063 are shRNAs used in the screen that target DDX5 expression whereas the other DDX5 shRNAs shown are new shRNAs not tested in the screen). B, colony formation results from the plasmid stability assay for either duplicate (each DDX5 shRNA)- or quadruplicate (Luciferasemi203 shRNA)-transduced cultures. C, colony formation results from the proliferation assay. Quantification of colony formation results are plotted in (B) and (C).
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DDX5 is involved in gene transcription (9). The observation that MCM2, MCM5, and CDC45 proteins are all downregulated in DDX5-depleted cells released from serum starvation suggests that DDX5 may regulate their expression. Indeed, knockdown of DDX5 in asynchronous HCT116 cultures was determined using flow cytometric analysis of propidium iodide incorporation and gating cells with greater than 2C but less than 4C DNA content. Western blot analysis of RB, G1-S cyclin expression, and DNA replication factor expression in G1 and early S-phase whole-cell extracts obtained from cells at increasing time following addition of serum to serum-starved cells previously transfected with the indicated siRNAs. Western blot analysis of replication factors in chromatin fractions obtained from siRNA-transfected S-phase cells over time after serum addition. The upper band detected on the CDC45 blots and marked with an asterisk in (D) and (E) is a nonspecific cross-hybridizing protein recognized by the antibody.

**Figure 3.** DDX5 is required for G1-S-phase progression. A, quantitative Western blot analysis of DDX5 and β-actin (loading control) in whole-cell extracts obtained from cultures 48 hours after transfection with the indicated siRNAs. B, flow cytometric analysis of cell cycle in 48-hour posttransfection cells with either DDX5si2008 (left) or EBNA1si1666 (right). C, progression into S-phase following serum addition to serum-starved cultures previously transfected with either DDX5si2008 (circles) or EBNA1si1666 (squares) siRNAs. The fraction of cells in S-phase at each time point after serum addition was determined using flow cytometric analysis of propidium iodide incorporation and gating cells with greater than 2C but less than 4C DNA content. D, Western blot analysis of RB, G1-S cyclin expression, and DNA replication factor expression in G1 and early S-phase whole-cell extracts obtained from cells at increasing time following addition of serum to serum-starved cells previously transfected with the indicated siRNAs. E, Western blot analysis of replication factors in chromatin fractions obtained from siRNA-transfected S-phase cells over time after serum addition. The upper band detected on the CDC45 blots and marked with an asterisk in (D) and (E) is a nonspecific cross-hybridizing protein recognized by the antibody.

DDX5 contributes toward DNA replication gene expression

DDX5 is required for G1-S-phase progression. A, quantitative Western blot analysis of DDX5 and β-actin (loading control) in whole-cell extracts obtained from cultures 48 hours after transfection with the indicated siRNAs. B, flow cytometric analysis of cell cycle in 48-hour posttransfection cells with either DDX5si2008 (left) or EBNA1si1666 (right). C, progression into S-phase following serum addition to serum-starved cultures previously transfected with either DDX5si2008 (circles) or EBNA1si1666 (squares) siRNAs. The fraction of cells in S-phase at each time point after serum addition was determined using flow cytometric analysis of propidium iodide incorporation and gating cells with greater than 2C but less than 4C DNA content. D, Western blot analysis of RB, G1-S cyclin expression, and DNA replication factor expression in G1 and early S-phase whole-cell extracts obtained from cells at increasing time following addition of serum to serum-starved cells previously transfected with the indicated siRNAs. E, Western blot analysis of replication factors in chromatin fractions obtained from siRNA-transfected S-phase cells over time after serum addition. The upper band detected on the CDC45 blots and marked with an asterisk in (D) and (E) is a nonspecific cross-hybridizing protein recognized by the antibody.
Figure 4. DDX5 is required for expression of DNA replication genes. A, quantitative Western blot analysis of DNA replication factors in whole-cell extracts obtained from asynchronous cell cultures 48 hours after siRNA transfection with the indicated siRNAs. The asterisks shown in the MCM2, MCM10, and CDC45 blots indicate the position of non-specific proteins detected by these antibodies. B, Q-PCR analysis of DNA replication factor transcript abundance in cells either 24 or 48 hours after transfection with DDX5si2008 (red bar), EBNA1si1666 (blue bar) or mock-transfected (no siRNA—green bar). Results for each transcript are normalized to the abundance of the indicated transcript in cells transfected with the EBNA1si1666 siRNA. Error bars indicate SDs calculated from 3 independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. C, heat map showing row-wise standardized expression level for DNA replication genes 24 hours after transfection of cells with the indicated siRNAs.

arising after DDX5 knockdown occurred before changes in cell-cycle progression, supporting that these gene expression changes contributed toward this phenotype.

DDX5 Is Required for RNA Polymerase II Recruitment to Promoters of DNA Replication Genes

We analyzed promoters of genes downregulated as a result of DDX5 knockdown for common transcription factor–binding motifs and found that the E2F-binding motif was frequently present (FDR = 4.55 × 10⁻⁶; Supplementary Fig. S7). Thus, we tested whether DDX5 interacted with the transcription factor E2F1 and indeed found an interaction between these 2 proteins (Fig. 5A). This interaction is direct, as purified glutathione S-transferase (GST)-E2F1 interacted with in vitro transcribed and translated DDX5 (Fig. 5A). We also tested whether DDX5 localized to promoters of the CDC6, CDC45, and MCM5 genes that contain E2F-binding sites and confirmed that CDC6 expression is E2F1-dependent in this cell line (Supplementary Fig. S8). We observed enrichment for DDX5 at these promoters compared with a non-E2F-regulated promoter (Fig. 5B); however, this interaction was not required for E2F1 to localize to these promoters, as knockdown of DDX5 did not impair E2F1 localization (Fig. 5C). These results suggested a direct role for DDX5 in DNA replication factor gene expression.

We investigated histone modifications and the abundance of proteins required for gene expression at DNA replication gene promoters. DDX5 knockdown did not reduce the abundance of either acetylated histone H3 or acetylated histone H4 at these promoters (Fig. 5D, data not shown). In contrast, DDX5 knockdown significantly reduced the abundance of both RNA polymerase II and TFIIIB at the DNA replication gene promoters (Fig. 5E and F). These results were consistent with the requirement of DDX5 for expression of these genes and indicated that DDX5 directly functions in transcriptional pre-initiation complex assembly at their promoters.

The DDX5 Locus Is Amplified in Breast Cancer

The gene expression data revealed a significant overlap between genes downregulated by DDX5 knockdown and...
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These observations prompted us to investigate whether DDX5 protein is frequently overexpressed in breast cancer combined with results from a recent study showing that the DDX5 expression (FDR = 0.08; with an OR of 2.12). We also extracted gene pathways that were correlated with DDX5 expression data for 533 breast tumors from The Cancer Genome Atlas (TCGA) database and analyzed this to identify gene pathways that were correlated with DDX5 expression, and found that genes annotated to the S-phase pathway in Reactome were significantly correlated with DDX5 expression (FDR = 0.08; with an OR of 2.12). Combined with results from a recent study showing that the DDX5 protein is frequently overexpressed in breast cancer (20), these observations prompted us to investigate whether the DDX5 gene was amplified in breast cancer. Our analysis included genome copy number data obtained from a previous study where representation oligonucleotide microarray analysis (ROMA) was applied to breast cancer samples obtained from the Karolinska Institute (Stockholm, Sweden) and the Oslo Micrometastasis Study (Oslo, Norway; ref. 32). We found that the DDX5 locus was amplified in 63 of 255 breast cancer genomes (Fig. 6A, red bar). We also analyzed breast tumor data available in the TCGA database for DDX5 copy number changes and observed DDX5 to be amplified in 26 of 93 tumors where DDX5 copy number changes were frequently co-amplified, as 37 of 63 DDX5-amplified breast cancer samples also had amplification of the ERBB2 locus (Fig. 6A, green bar). The P value from Fisher exact test for this degree of coincidence was 3.9 × 10^{-15}. Although the ERBB2 and DDX5 genes described as a proliferation cluster in breast cancer (FDR = 8.3 × 10^{-15}; ref. 30). Proliferation cluster genes are those whose expression is positively correlated with the mitotic index of a given breast tumor sample. Most of the genes shared between these 2 sets of expression data were downregulated in DDX5-depleted cells, supporting the idea that DDX5 was required for expression of genes that promoted cell proliferation and suggested a connection between DDX5 and breast cancer cell proliferation. We also extracted expression data for 533 breast tumors from The Cancer Genome Atlas (TCGA) database (31) and analyzed this to identify gene pathways that were correlated with DDX5 expression, and found that genes annotated to the S-phase pathway in Reactome were significantly correlated with DDX5 expression (FDR = 0.08; with an OR of 2.12). Combined with results from a recent study showing that the DDX5 protein is frequently overexpressed in breast cancer (20), these observations prompted us to investigate whether the DDX5 gene was amplified in breast cancer. Our analysis included genome copy number data obtained from a previous study where representation oligonucleotide microarray analysis (ROMA) was applied to breast cancer samples obtained from the Karolinska Institute (Stockholm, Sweden) and the Oslo Micrometastasis Study (Oslo, Norway; ref. 32). We found that the DDX5 locus was amplified in 63 of 255 breast cancer genomes (Fig. 6A, red bar). We also analyzed breast tumor data available in the TCGA database for DDX5 copy number changes and observed DDX5 to be amplified in 26 of 93 tumors where DDX5 copy number changes were frequently co-amplified, as 37 of 63 DDX5-amplified breast cancer samples also had amplification of the ERBB2 locus (Fig. 6A, green bar). The P value from Fisher exact test for this degree of coincidence was 3.9 × 10^{-15}. Although the ERBB2 and DDX5...
genes are both present on chromosome 17, we saw many instances where they were amplified as separate, independent amplicons (e.g., Fig. 6A). This was consistent with the correlation between DDX5 overexpression and ERBB2 expression previously reported in breast cancer (10). Subtype information was available for the subset of 104 tumors collected from the Oslo Micrometastasis Study, and we found DDX5 amplification in each subtype with the exception of the “normal” unclassified subtype (Fig. 6C). Because DDX5 is frequently amplified in ERBB2-positive breast tumors, we tested whether DDX5 knockdown affected the sensitivity of ERBB2-positive breast cancer cells to trastuzumab, and we found that DDX5 knockdown increased the sensitivity of these cells to inhibition of proliferation by trastuzumab (Fig. 6D; Supplementary Fig. S9A–S9C).

Breast Cancer Cells with Amplification of DDX5 Are More Sensitive to Its Depletion than Breast Cancer Cells Lacking Amplification of DDX5

We were interested in testing whether there were differences in the sensitivity of breast cancer cells to DDX5 depletion for DDX5-amplified versus nonamplified cell lines, and so we identified breast cancer cell lines with or without amplification of the DDX5 locus (Supplementary Fig. S10A and S10B). The sensitivity of 4 different breast cancer cell lines with amplification of the DDX5 locus to DDX5 depletion by 3 different potent shRNAs was tested and significant inhibition of proliferation was observed (Fig. 7A). Knockdown efficiency of DDX5 in the different cell lines varied. The strongest knockdown of DDX5 was achieved in the MDA-MB-453 and SK-BR-3 cell lines where 90% knockdown of DDX5 protein was observed, resulting in 5- to 10-fold inhibition of cell proliferation. In contrast, when we transduced 3 different breast cancer cells lacking amplification of the DDX5 locus and the breast epithelial cell line, MCF10A, with the DDX5 shRNAs, none of the DDX5 shRNAs impaired their proliferation 2-fold or greater despite achieving 90% or more knockdown of DDX5 in each of these cell lines (Fig. 7B). We conclude that breast cancer cells having amplification of the DDX5 gene were more dependent upon its expression to proliferate than breast cancer cells lacking this amplification.

Because DDX5 promoted expression of DNA replication factors in HCT116 cells, we tested how DDX5 depletion affected the expression of DNA replication genes in the DDX5-amplified breast cancer cell line SK-BR-3 versus the DDX5-nonamplified cell lines HCC1143 and MCF10A and observed that DDX5 depletion led to downregulation of DNA replication proteins.
Figure 7. Differential sensitivity of breast cancer cells to DDX5 depletion. A, quantitative Western blot analysis of DDX5 and β-actin and colony formation assay results for different breast cancer cell lines with amplification of the DDX5 locus following transduction of the indicated DDX5 shRNAs or EBNA1 shRNA (negative control). Representative colony formation assay cultures are shown. Plots reflect quantitated results for the colony formation assays. Error bars show SDs for triplicate cultures. B, same as in (A) except that breast cancer cell lines lacking DDX5 amplification are tested. C, Western blot (WB) analysis of DNA replication factors in the indicated cell lines transduced with either DDX5mi2008 (“D5”) or EBNA1mi1666 (“E”). The asterisk indicates a nonspecific cross-hybridizing band detected by the CDC45 antibody in the SK-BR-3 and MCF10A whole-cell extracts. These results were analogous to observations in HCT116 cells and support the conclusion that DDX5 downstream of RB and E2F to elevate DNA replication gene expression in breast cancer cell lines with amplification of the DDX5 locus following transduction of the indicated DDX5 shRNAs or EBNA1 shRNA (negative control). Representative colony formation assay cultures are shown. Plots reflect quantitated results for the colony formation assays. Error bars show SDs for triplicate cultures. D, Q-PCR analysis of the indicated transcripts in SK-BR-3 cells transduced with either DDX5mi2008 (red bars) or EBNA1mi1666 (blue bars). Results are normalized to the relative abundance of the indicated transcripts in the EBNA1mi1666-transduced cultures. Error bars indicate the SDs from duplicate experiments. E, Q-PCR analysis of RNA polymerase II ChIP samples obtained from SK-BR-3 cells transduced with either DDX5mi2008 (red bars) or EBNA1mi1666 (blue bars). Results presented are normalized to the relative abundance of the indicated promoters in ChIP samples obtained from EBNA1mi1666-transduced cultures. Error bars indicate the SDs from duplicate experiments.

We found that DDX5 is required for plasmid stability and G1-S-phase progression in a colorectal cancer cell line that overexpresses DDX5 where it regulates DNA replication by directly promoting the expression of DNA replication genes. DDX5 depletion impairs DNA replication factor gene expression in the absence of an effect on either RB phosphorylation or E2F1 localization to their promoters, indicating a role for DDX5 downstream of RB and E2F to elevate DNA replication factor gene expression and enhance cell proliferation. DDX5 is required for the general transcription factor TFIIB and RNA polymerase II to localize to the promoters of genes encoding DNA replication factors showing an activity in the early steps of transcription initiation at these promoters. This mirrors the observations of others who studied transcriptional activation of promoters regulated by estrogen receptor-α and MyoD (33, 34). In the former study, DDX5 localized to the pS2 promoter in response to estradiol treatment and before TFIIB and RNA polymerase II recruitment. In the latter study, DDX5 was required for the localization of the chromatin remodeler, BRG1, as well as the general transcription factor, TBP, and RNA polymerase II to both the MHCIIIB and TNNC2 promoters following induction of C2C12 cell differentiation. Consistent with this latter study,
we did not observe an effect of DDX5 knockdown on histone acetylation at DDX5-dependent promoters, indicating that DDX5 does not play an essential role in HAT recruitment.

DDX5 was initially identified as a host protein that cross-reacted against an antibody raised against SV40 large T-antigen (7). This similarity between DDX5 and large T may mark a conserved activity, as large T has also been shown to function in transcriptional pre-initiation complex assembly at gene promoters (35, 36). Our results suggest that relief of RB suppression alone may not be sufficient to upregulate E2F-dependent expression of DNA replication factor genes and hence cell proliferation. Consistent with this, HCT116 cells that overexpress DDX5 also lack wild-type p16INK4a expression, which antagonizes RB phosphorylation by cyclin D-CDK4, and our analysis of gene expression data for 533 breast tumors available from the TCGA database revealed that DDX5 expression in breast cancer is correlated with cyclin D1 expression ($P = 2.38 \times 10^{-6}$) and is inversely correlated with p16INK4a expression ($P = 5.67 \times 10^{-5}$). This indicates that the proliferative advantage conferred by DDX5 overexpression to tumors occurs in the presence of mutations that impair the RB pathway. We suggest that amplification or overexpression of DDX5 leads to increased transcription via a new mechanism that is downstream of RB regulation. Such deregulation most likely requires interaction of DDX5 with the gene promoters because we have localized it to these gene regulatory elements and it is required for efficient loading of TFIIIB and RNA polymerase II. Recently, an essential role for ncRNAs in the regulation of E2F-dependent gene expression has been shown (37). We propose that DDX5 may couple this ncRNA activity or the activity of another ncRNA at E2F promoters with RNA polymerase II recruitment to promote gene expression in the absence of repression by RB.

The activity of DDX5 in promoting DNA replication gene expression is cell context–dependent, as it contributes toward the expression of these genes only in cell lines where it is either overexpressed (HCT116) and/or amplified (SK-BR-3). DDX5 knockdown did not affect the expression of DNA replication factors in either the HCC1143 or the MCF10A cell lines in which the DDX5 gene is not amplified. It is possible that the activity of DDX5 at DNA replication promoters is redundant with another protein where this redundancy is lost in cancer cells with DDX5 overexpression/gene amplification. Alternatively, DDX5 may not normally function in DNA replication gene expression but this activity may be acquired during tumorigenesis, perhaps oncogene-stimulated. Consistent with the latter, DDX5 interacts with E2F1 in the DDX5 amplification cell lines MDA-MB-453 and SK-BR-3 cells but not in MCF10A cells. These results point toward the positive role for DDX5 in DNA replication factor gene expression being acquired during cancer development, where it upregulates expression of these genes and confers a significant advantage to cancer cell proliferation. Importantly, our data suggest that cancer cells then become dependent on DDX5 expression, a vulnerability that cancer cells support a broad dependence of breast cancers on DDX5 activity and underscore the need to understand whether this activity can be targeted by pharmacologic inhibitors. Moreover, as depletion of DDX5 enhances the sensitivity of ERBB2-positive breast cancer cells to trastuzumab, an inhibitor against DDX5 may enhance the efficacy of trastuzumab in breast cancer therapy. Cyclin D is essential for neu-driven tumorigenesis in mouse models and for proliferation of ERBB2-positive breast cancer cells (38, 39). Combination treatment of ERBB2-amplified breast cancer cells with flavopiridol, a cyclin-dependent kinase inhibitor, and trastuzumab results in synergistic inhibition of proliferation (40). Cyclin D-CDK4 hyperphosphorylates RB to de-repress E2F-dependent gene expression and inhibition of cyclin D-CDK4 should restore RB-mediated repression of these genes. We propose that synergistic inhibition of cell proliferation resulting from DDX5 knockdown and trastuzumab treatment follows a similar mechanism as cyclin D inhibition where DDX5 depletion impairs RNA polymerase II recruitment to E2F-regulated promoters and thus antagonizes E2F-dependent gene expression.

We observed frequent amplification of DDX5 in luminal subtype breast cancers consistent with the previously described activity of DDX5 as a transcriptional co-activator of estrogen receptor-α–dependent gene expression (41). We also observed frequent co-amplification of the ERBB2 and DDX5 genes. This agrees with the significant correlation reported for ERBB2 and DDX5 expression in a panel of estrogen receptor-α–positive breast tumors (20). However, our analysis of the ERBB2/DDX5 double-positive breast cancers did not reveal a correlation with estrogen receptor expression and thus suggests an estrogen receptor–independent activity for DDX5 in breast cancer. Indeed, the SK-BR-3 and MDA-MB-453 breast cancer cell lines we found to be dependent upon DDX5 to proliferate are negative for estrogen receptor expression. Interestingly, in addition to identifying a significant correlation between DDX5 and ERBB2 expression in their panel of estrogen receptor-α–positive breast tumors (20), we also observed a significant correlation between DDX5 and AIB1 (NCOA3) expression. NCOA3 has been shown to be a transcriptional co-activator of E2F-regulated genes (42, 43). In light of our results, we suggest that DDX5 and NCOA3 may cooperate in breast cancer to upregulate the expression of DNA replication genes and thus promote cancer cell proliferation.

Our findings that the DDX5 gene is frequently amplified in breast cancer and that breast cancer cell lines harboring this amplification are more sensitive to its depletion than breast cancer cell lines that lack DDX5 amplification suggest that DDX5 may function as an oncogene. Overexpression of DDX5 in mouse fibroblasts promotes transformation and tumor formation in nude mice (44). However, we have been unable to overexpress a DDX5 transgene in many different human and murine cancer and non-cancer cell lines (see Supplementary Fig. S5A–S5C). This has hampered our efforts to test whether elevated DDX5 expression transforms breast epithelial cells and also to identify mutants in an RNAi-resistant DDX5 transgene that do not restore cell proliferation to DDX5-dependent cell lines with endogenous DDX5 knockdown. We speculate
that co-expression of DDX5 with another protein and/or ncRNA may enable robust expression of the DDX5 transgene and we are currently investigating this hypothesis.

The data herein suggest that DDX5 is a viable candidate drug target for selective anti-cancer therapy directed at those tumors that have an amplified DDX5 locus. We are currently testing this notion by conducting a screen for inhibitors of DDX5 activity. Like treatment with trastuzumab that is testing this notion by conducting a screen for inhibitors of HER2 activity, cancer treatment targeting DDX5 could be linked to those breast cancers that have this locus amplified.

**METHODS**

A detailed description of materials and methods is provided in Supplementary Material.

**Antibodies**

Western blot analysis: anti-DDX5 cat. #A300-523A, anti-DDX17 cat. #A300-509A, anti-MCM5 cat. #A300-195A, anti-AND1 cat. #A300-605A, anti-MCM10 cat. #A301-141A, and anti-NCP-A2 cat. #A300-605A (Bethyl Laboratories); anti-β-actin cat. #A5316, anti-PCNA cat. #P8825, and anti-E2F1 cat. #E8901 (Sigma); anti-cDC45L cat. #ab56746 (Abcam); anti-CCND1 cat. #2922 and anti-CCNE2 cat. #4132 (Cell Signaling); anti-CCNA2 cat. #NB100-91726 (Novus); anti-RB cat. #554136 (Pharmingen); and anti-MCM10 cat. #12251-1-AP (Proteintech Group).

In-house antibodies that were used include anti-MCM2 #C732 (polyclonal), anti-MCM3 #738 (polyclonal), anti-cDC6 #1881 (polyclonal), anti-ORC1 clone PKS 1-40 (monoclonal), anti-ORC6 clone 30 (monoclonal), anti-ORC2 pAb205B (polyclonal), anti-ORC3 PKS16-11 (monoclonal), anti-RB C-36 (monoclonal), and anti-RB X2-55 (monoclonal). The antibody against PSF2 was kindly provided by Dr. Juan Mendez (CNIO, Madrid, Spain). For immunoprecipitation experiments, rabbit anti-DDX5 (Bethyl Laboratories cat. #A300-523A) and normal rabbit anti-IgG (CalTag Laboratories cat. #10508C) were used. For chromatin immunoprecipitation (ChIP) experiments, rabbit anti-DDX5 (Bethyl Laboratories cat. #A300-523A), mouse anti-E2F1 (Sigma cat. #E8901), rabbit anti-acetyl-Histone H3 (Millipore cat. #06-599), rabbit anti-RNA polymerase II (Santa Cruz Biotechnology cat. #sc-899), and rabbit anti-TFIIIB (Santa Cruz Biotechnology cat. #sc-225X) were used.

**Cell Lines and Growth Media**

HCT116 cells were provided by Dr. Richard Boland (Baylor University Medical Center, Dallas, TX) and propagated in Dulbecco’s Modified Eagle Media (DMEM; Cellgro #10-013-CV) + 10% FBS. SK-BR-3, MDA-MB-453, EVSA-T, ZR-75-1, and HCC1143 cells were provided by Dr. Richard Boland (Baylor University Medical Center, Dallas, TX) and propagated in McCoy’s (Cellgro #10-050-CV) + 10% FBS (SK-BR-3), Leibovitz (ATCC #30-2008) + 10% FBS (MDA-MB-453), MEM (ATCC #30-2003) + 2 mmol/L L-glutamine + 10% FBS (EVSA-T), or RPMI-1640 (Cellgro #10-040-CV) + 10% FBS (HCC1143 and ZR-75-1), respectively. EFM-19 and HS578a cells were provided by Dr. Michael Wigler (Cold Spring Harbor Laboratory) and were propagated in RPMI-1640 + 10% FBS and DMEM + 10 μg/mL insulin + 10% FBS, respectively. MCF10A cells were provided by Dr. Senthil Muthuswamy (Cold Spring Harbor Laboratory) and propagated in DMEM/F12 (Gibco #11330) + 20 ng/mL EGF + 500 ng/mL hydrocortisone + 100 ng/mL cholera toxin + 10 μg/mL insulin + 5% horse serum. No authentication of the cell lines was conducted by the authors.

**Plasmids**

All shRNAs are designed in a mir30 backbone and were either obtained from Open Biosystems (indicated as V2HS_xxxxxx in the text) or were self-prepared (indicated as GeneNamexxxxxx in the text). The p220.2 plasmid encoding OriP, EBNA1, and hygromycin resistance was kindly provided by Dr. Anindya Dutta (University of Virginia; ref. 4).

**Screen**

HCT116 cells (150,000 per well) were seeded in 6-well plates and allowed 24 hours to attach. They were then infected with the 36- and 48-hour viral supernatant (see Supplementary Material for packaging of retrovirus encoding shRNAs) for a total of 2 rounds of infection per culture. Twenty-four hours after the second round of infection, cells from each well were suspended and seeded into new wells of 6-well tissue culture plates in DMEM + 10% FBS + 1.5 μg/mL puromycin. Cultures were then selected for 4 days and then were suspended by trypanoscopy and counted using a hemacytometer. Cells (600,000) from each suspension were then seeded to separate wells of 6-well plates in 2 mL DMEM + 10% FBS per well. Following 24 hours, each culture was transfected with 1 μg of p220.2 plasmid (encoding OriP, EBNA1, and hygromycin resistance) diluted in Opti-Mem, 4 μL Lipofectamine (Invitrogen #18324-012), and 6 μL Plus Reagent (Invitrogen #11514-015) using the manufacturer’s protocol. Transfected cultures were then incubated in a cell culture incubator (37°C, 5% CO2) for 4.5 hours. The transfection media were then aspirated, each culture was washed with PBS, then 2 mL DMEM + 10% FBS was added to each culture before returning to the incubator. Twenty-four hours after p220.2 plasmid transfection, cells were suspended by trypanoscopy and counted using a hemacytometer. For the proliferation assay, 1 well of a 12-well tissue culture plate was seeded with 5,000 cells per suspension in 1 mL DMEM + 10% FBS + 1.5 μg/mL puromycin. For plasmid stability assay, 1 well of a 12-well tissue culture plate was seeded with 50,000 cells per suspension in 1 mL DMEM + 10% FBS + 1.5 μg/mL puromycin + 400 μg/mL hygromycin. Selection media were replaced every third day. Colonies in proliferation assay cultures were expanded for 8 days and plasmid stability assay cultures for 10 days before staining with crystal violet.

The plasmid stability ratio for each experimental shRNA was calculated by dividing the plasmid stability result (see colony formation assay description in Supplementary Materials and Methods) for the culture transduced with the experimental shRNA by the proliferation result for the culture transduced with the same experimental shRNA.

**Serum Starvation and Chromatin Fractionation Experiments**

HCT116 cultures seeded with 125,000 cells per well of 6-well plates and allowed 24 hours to attach were transfected with the indicated siRNAs as described in Supplementary Material. Twenty-four hours following transfection, the media were removed, and each culture washed twice with 2 mL DMEM (no serum) per culture. The cultures were then incubated for 48 hours in DMEM lacking serum. Following the incubation, the serum-free media were removed from each culture and replaced with DMEM + 10% FBS. Chromatin fractionation was conducted at the indicated time points after serum addition as described (45).

**Quantitative PCR Analysis**

RNA was prepared from siRNA-transfected HCT116 cultures at the indicated time points after siRNA transfection using the RNeasy Mini Kit (Qiagen cat. #74014) including on-column DNase digestion (Qiagen cat. #79254) and eluted in the supplied RNase-free water. RNA from shRNA SK-BR-3 cultures was prepared from cells 8 days after siRNA transfection. The cDNA used for quantitative PCR (Q-PCR) was prepared from 1 μg of each RNA sample using TaqMan Reverse Transcriptase Reagents (Applied Biosystems #N808-0234) with random hexamer priming in a GeneAmp PCR system 9700 thermocycler. Each Q-PCR reaction was prepared with 2 μL of 1:20 diluted cDNA and 13 μL LightCycler 480 SYBR Green I Master Mix.
Microarrays and Analysis

RNA was isolated from HCT116 cultures 24 hours after siRNA transfection. Preparation of cDNA, sample labeling, sample hybridization to microarrays, and microarray scanning were conducted by the Microarray Shared Resource Facility at Cold Spring Harbor Laboratory (Cold Spring Harbor, NY). Samples were hybridized to the Affymetrix GeneChip Human Gene 1.0 ST Array. Raw data from the arrays were then processed using the robust multiarray averaging (RMA) method with an up-to-date probe set definition (46, 47). The latest gene annotation information was retrieved from the Gene Ontology public database. Microarray data have been deposited in NCBI’s Gene Expression Omnibus and is accessible through GEO series accession number GSE36141 (ref. 48; accession number GSE36141). Differential expression, gene set, and transcription factor motif analysis are presented in the Supplementary Information.

Nuclear Extract Preparation, Immunoprecipitation, GST Interaction Assay, and Chip Assay

Descriptions are presented in the Supplementary Information.

Analysis of DDX5 Copy Number in Breast Tumors and Copy Number versus Expression in Breast Cancer Cell Lines

Genomic DNA was prepared from 33 breast cancer cell lines, and copy number analysis was conducted in these cell lines relative to a normal reference cell line using ROMA as described (32). Gene expression profiling was conducted using NimbleGen Gene Expression arrays using conditions recommended by the manufacturer. Quantile normalization intensities obtained from the arrays were converted to log2 ratios by comparing results obtained from the breast cancer cell lines with the universal reference RNA.

Segmented DNA copy number profiles of chromosome 17 were generated from publicly available normalized copy number ratio data for the MicMa set (125 profiles) and for the WZ set (141 profiles) of breast tumor tissues (refs. 32, 48, 49; accession number GSE19425). The segmentation was conducted as described (32).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conception and design: A. Mazurek, W. Luo, B. Stillman
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Mazurek, J. Hicks, R.S. Powers
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Mazurek, W. Luo, A. Krasnitz, J. Hicks, R.S. Powers, B. Stillman
Writing, review, and/or revision of the manuscript: A. Mazurek, W. Luo, B. Stillman

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Mazurek, R.S. Powers, B. Stillman

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