The Atypical Histone MacroH2A1.2 Interacts with HER-2 Protein in Cancer Cells*

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Background: HER-2/c-erbB-2 is commonly overexpressed in cancers, but how its overexpression is achieved is not well understood.

Results: HER-2 was found to interact with the atypical histone macroH2A1.2.

Conclusion: HER-2 cooperates with macroH2A1.2 to drive HER-2 overexpression in cancer cells.

Significance: The discovery of a novel interaction between mH2A1.2 and HER-2 reveals a unique mechanism by which oncogenes can broadly deregulate gene transcription in cancer cells.

Because HER-2 has been demonstrated in the nuclei of cancer cells, we hypothesized that it might interact with transcription factors that activate ERBB2 transcription. Macrohistone 2A1 (H2AFY; mH2A1) was found to interact with HER-2 in cancer cells that overexpress HER-2. Of the two human mH2A1 isoforms, mH2A1.2, but not mH2A1.1, interacted with HER-2 in human cancer cell lines. Overexpression of mH2A1.2, but not mH2A1.1, in cancer cells significantly increased HER-2 expression and tumorigenicity. Inhibition of HER-2 kinase activity diminished mH2A1 expression and mH2A1.2-induced ERBB2 transcription in cancer cells. Chromatin immunoprecipitation of mH2A1.2 in cancer cells stably transfected with mH2A1.2 showed enrichment of mH2A1.2 at the HER-2 promoter, suggesting a role for mH2A1.2 in driving HER-2 overexpression. The evolutionarily conserved macro domain of mH2A1.2 was sufficient for the interaction between HER-2 and mH2A1.2 and for mH2A1.2-induced ERBB2 transcription. Within the macro domain of mH2A1.2, a trinucleotide insertion (-EIS-) sequence not found in mH2A1.1 was essential for the interaction between HER-2 and mH2A1.2 as well as mH2A1.2-induced HER-2 expression and cell proliferation.

HER-2 overexpression occurs in several types of cancers, including breast, colorectal, gastric, esophageal, and pancreatic, and mediates cancer cell invasion, metastasis, survival, and proliferation (1–6). HER-2 overexpression portends poor survival in patients (1, 7–13). HER-2 is a member of the EGFR family, but unlike the other members (EGFR/HER-1, HER-3, and HER-4), which possess cognate ligands, it is an orphan receptor. However, HER-2 can mediate signal transduction through heterodimerization with the other members of the EGFR family, which can transactivate HER-2 tyrosine kinase activity (14).

Although EGFR/c-erbB family members are best known as membrane receptor tyrosine kinases, they have been observed in the nuclei of cells (15–23). A previous study demonstrated phosphorylated HER-2 in the nuclei of cancer cells where it activated COX-2 gene transcription by binding to the COX-2 gene promoter (23). Another group showed that HER-2 forms a transcriptionally active complex with the transcription factor STAT3 (24). These studies suggest a role for HER-2 in transcriptional deregulation in cancer cells. We hypothesized that HER-2 may effect its own overexpression through interactions with transcription factors that interact with and activate transcription of the ERBB2 promoter. We now show that HER-2 interacts with the atypical histone macrohistone 2A1.2, and this interaction is important for HER-2 overexpression in cancer cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Colon cancer (Caco-2) and HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium. Ovarian (SKOV-3) and breast (SKBR-3) cancer cells were cultured in McCoy’s medium. All media were supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin. All cells were grown at 37 °C in 5% CO2 unless otherwise stated. Transient transfection was carried out using the lipofection reagent FuGENE (Roche Applied Science) as described previously (17). Stable transfection of SKOV-3 and SKBR-3 cells was carried out in 500 μg/ml G418-containing medium as previously described (17).

Antibodies—Trastuzumab (anti-HER-2 mAb) was obtained from the Huntsman Cancer Institute pharmacy. HER-2 monoclonal antibody (mAb) AB-3 (Oncogene Research), biotinylated HER-2 mAb (LabVision), macroH2A1 polyclonal anti-
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...body (Millipore/Upstate), V5 mAb (Invitrogen), MYC polyclonal antibody (Roche Applied Science), HRP-conjugated phosphotyrosine mAb 4G10 (Upstate), phospho-HER-2 mAb (Cell Signaling Technology), and horseradish peroxidase-conjugated goat anti-mouse and rabbit anti-mouse (Zymed Laboratories Inc.) were obtained from the sources indicated.

Expression Constructs—MacroH2A1.1-CT-MycHis/pcDNA3.1 and macroH2A1.2-CT-MycHis/pcDNA3.1 were kind gifts from Dr. Brian P. Chadwick (Duke Institute for Genome Sciences and Policy). Full-length human ERBB2 was subcloned into pcDNA3.1 (pcDNA3.1-HER-2) as described previously (17). The SV40 large T antigen nuclear localization sequence (NLS) (5'-AGGCCAAAGAAGAAGAAAGATAGAAA-3') was synthesized with XhoI and XbaI restriction endonuclease sites at the 5'- and 3'-ends, respectively, allowing it to be ligated in-frame with the C-terminal end of macroH2A1.2 (in pcDNA3.1) using the XhoI and XbaI restriction sites.

HALO® Epitope-tagged Constructs—Full-length macrohistone 2A1.2 (mH2A1.2) was excised from mH2A1.2-CT-MycHis/pcDNA3.1 with EcoRV/HindIII, then end-filled, and ligated into EcoRV-treated pHT2. The mH2A1.2 macro domain was excised from macroH2A1.2-CT-MycHis/pcDNA3.1 with BamHI and ligated into the BamHI site of pHT2. The Kozak sequence was eliminated from macroH2A1.2-CT-MycHis/pcDNA3.1 using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions and the following primers: 5'-GGATATCCGGCACCGTAATCCACTA-3' and 5'-TGGATGTTCCAGCCATATCC-3'.

V5/His6 Epitope-tagged mH2A1 Constructs—Full-length mH2A1.1 and mH2A1.2 were excised with Nhel/HindIII from macroH2A1.1-CT-MycHis/pcDNA3.1 and macroH2A1.2-CT-MycHis/pcDNA3.1, respectively, and subcloned into pcDNA4 (Invitrogen). The macro domain of mH2A1.2 was excised with Nhel/EcoRI from macro-HALO®/pHT2 and ligated into the Nhel/EcoRI sites in pcDNA4. The H2A2 fragment was excised from mH2A-CT-MycHis/pcDNA3.1 with BamHI and ligated into the BamHI site in pcDNA4.

mH2A1.2 Mutant Constructs—The QuikChange site-directed mutagenesis kit (Stratagene) was used according to the manufacturer’s instructions to generate the following mutations and deletion of the macro domain in pcDNA4-mH2A1.2 with the following primers: E202A/S204A mutant, 5'-CTGACCTTATCCAGTCTAATCCCTGTTAGG-3' and 5'-CTCAAAACCGGCTAATTACCGATTAGACTGTAATAGGTCC-3'; Δ31 deletion mutant, 5'-GGACACCGCCGACCGGCCTATACATCCATCCTA CCA-3' and 5'-TGGTAGATTGATTGCGCGCCTCGGCA GGTGTC-3'.

Tandem Affinity Purification and Mass Spectrometry—The full-length human ERBB2 construct from pcDNA3.1-HER-2 was subcloned into the XhoI site of the tandem affinity purification vector pNTAP (Stratagene). The pNTAP-HER-2 construct was transiently transfected into HEK 293 cells. Forty-eight hours later, the cells were lysed according to the manufacturer’s instructions. After normalization for total protein concentration, the lysates were incubated with streptavidin beads overnight at 4°C. The beads were washed one or four times and then boiled in SDS reducing sample buffer. The proteins were resolved on 7.5% SDS-polyacrylamide gels and silver-stained. Bands of interest were cut out of the gels, incubated in trypsin (Promega) overnight, and purified by reverse-phase chromatography on C18 ZipTips® (Millipore).

Mass spectrometric identification of proteins was performed as described previously (21). Briefly, trypsin digestions were analyzed using positive ion electrospray ionization LC-MS/MS analysis in an Eksigent Nano LC-1D binary pump HPLC system interfaced to a Finnigan LCQ Deca ion trap mass spectrometer (ThermoElectron Corp.) equipped with a Picoview Nanospray® source. The scan range for MS mode was set at m/z 400–1800 Da. Identified peptides from protein digestes were assigned from MS/MS and NCBI protein database searches using the Mascot search engine (Matrix Science, Inc.).

Quantitative RT-PCR—Total RNA was isolated from cells using an RNaseasy kit (Qiagen). First strand cDNA was synthesized from 1 µg of total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed using SYBR Green incorporation on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems). Threshold cycles for SYBR Green primers were normalized to G3PDH. The Tm was 80–84°C. PCR was carried out using the following set of primers for ERBB2: 5'-TGAGGACCTGGAAGGTTGC-3' and 5'-TGGATGTCAGGCAGA TGC-3'.

Immunoblotting—Cells cultured on 100-mm plastic dishes were extracted at various times after plating in lysis buffer, sonicated for 10s, and clarified at 14,000 rpm at 4°C. Protein concentrations were determined by the Bradford protein assay. 50–100 µg of total protein for each lysate or immunoprecipitate were boiled in reducing sample buffer for 3 min and then resolved on 7.5 or 4–20% Tris-HCl SDS-polyacrylamide gels by electrophoresis. The gels were transferred to nitrocellulose and blocked overnight in blocking buffer B (1% bovine serum albumin, 100 mM Tris-Cl, pH 7.4, 0.9% NaCl, and 0.1% Nonidet P-40). The blots were then incubated with primary antibodies in blocking buffer B at 4°C overnight. The blots were washed twice in blocking buffer B for 20 min each and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at 4°C. The blots were washed twice in blocking buffer B for 20 min each, incubated with SuperSignal™ horseradish peroxidase substrate (Pierce) for 1 min, and then exposed to film. Densitometry was performed on the bands on the blots using NIH Image.
samples were then used to perform immunoblotting as described above.

**Cell Proliferation Assay**—Cells were dispersed and plated at 40,000 cells/well in 96-well dishes. At various days in culture, the cells were gently washed twice with 100 μl/well ice-cold PBS. The cells were fixed for 10 min in 100% ice-cold methanol (100 g/l) and then air-dried. The stained cells were then solubilized in 1% sodium deoxycholate, and the plates were read at 590 nm in a spectrophotometer. The absorption at 590 nm is proportional to the number of attached cells.

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**Athymic Mouse Xenograft Model**—Xenografts of 106 SKOV-3 cells stably transfected with vector or macroH2A1.2 were generated as described previously (22). Briefly, the cancer cells were harvested in 0.25% trypsin, PBS, and EDTA; washed once in medium and PBS; and resuspended in medium at 1 million cells/200 μl. One million cells were injected subcutaneously in the backs of 5-week-old female nude athymic mice (Charles River Laboratories). Subcutaneous tumor sizes were determined by measuring the length and width with calipers, and the tumor volumes were calculated as the length × width². The mean sizes of the tumors were compared using the Mann-Whitney test. All animal studies presented were done with the approval of and in accordance with the University of Utah Institutional Animal Care and Use Committee.

**Luciferase construct**—The ERBB2 promoter-firefly luciferase construct (HER2-promoter-luciferase/RO6) was a gift from Dr. Chris Benz (Buck Institute) and consists of 0.5 kb of the ERBB2 promoter subcloned into the pGL3 vector. HER2-promoter/RO6 and Renilla luciferase (a gift from Dr. Nadeem Moghal, Huntsman Cancer Institute) constructs (2:1 ratio) were transiently co-transfected into HEK 293 cells with Lipofectamine 2000 (Invitrogen). 24 h later, the cells were rinsed with PBS, lysed at RT for 20 min in luciferase assay lysis buffer (0.1% Triton X-100, 25 mM Gly-Gly (pH 7.8), 15 mM MgSO₄, 4 mM EGTA, and 1 mM DTT), and then centrifuged at 4 °C for 15,000 g and the resulting supernatants were collected. For detection of Renilla luciferase activity, 20 μl of supernatant were placed in a 96-well microtiter plate followed by injection of 100 μl of coelenterazine substrate (120 nM coelenterazine in PBS). For detection of firefly luciferase activity, 100 μl of reaction buffer (25 mM Gly-Gly (pH 7.8), 15 mM MgSO₄, 4 mM EGTA, 37.5 mM potassium phosphate (pH 7.8), 2 mM DTT, and 2.5 mM ATP) per well were placed in a microtiter plate. Then 50 μl of cell supernatant were added along with luciferin substrate (150 μg/ml d-luciferin, 25 mM Gly-Gly (pH 7.8), 15 mM MgSO₄, and 4 mM EGTA). Luciferase activity was detected in a luminometer. ERBB2 promoter-(firefly) luciferase

**Figure 1.** HER-2 interacts with the atypical histone macrohistone 2A1. A, silver stain of SDS-PAGE showing TAP-HER-2 (arrowhead) and proteins co-purified with TAP-HER-2 using streptavidin beads. B, persistence of a 42-kDa protein (arrow) eluted from streptavidin beads with biotin (bio) after pull-down of TAP-HER-2 with streptavidin beads in a silver-stained SDS-polyacrylamide gel. C and D, immunoprecipitation (IP) of HER-2 was performed using antibodies specific for unphosphorylated (C) and phosphorylated (D) HER-2 from lysates containing equal total protein concentrations. Western (W) blots for HER-2, phospho-HER-2 (p-HER-2), and mH2A1 were then performed (C and D). Each of the results shown above is typical of two to three separate experiments. E, Western blots of mH2A1 and HER-2 generated from lysates of SKBR-3 and SKOV-3 cells that had been treated with 10 μg/ml anti-HER-2 mAb for various times. F and G, Caco-2 cells were treated with anti-HER-2 mAb for 24 – 48 h after which they were lysed and normalized for total protein concentration or used for RNA extraction and purification. Western blots for phospho-HER-2, mH2A1, and actin (F) and quantitative RT-PCR for mH2A1 mRNA levels (G) were performed. Error bars represent the standard error of the means. *, p < 0.05 compared with control.
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readings were normalized to Renilla values to account for variations in transfection efficiency of the reporter constructs.

Chromatin Immunoprecipitation—ChIP experiments were performed using the EZ ChIP™ chromatin immunoprecipitation kit (Millipore catalog number 17-371) according to the manufacturer’s protocol. Immunoprecipitation was performed with mH2A1 antibody (US Biological catalog number H5110-08L). Rabbit IgG (Abcam, ab46540) was used as an isotype control antibody. The chromatin-antibody complexes were captured on salmon sperm DNA/Protein G-Agarose (Upstate). After washing and elution of the complexes from the beads, the DNA-protein cross-links were reversed at 65 °C overnight. The immunoprecipitated DNA was treated with RNase A and proteinase K and purified using phenol-chloroform extraction and ethanol precipitation. Input DNA starting from aliquots of cell lysates was purified using phenol-chloroform extraction and ethanol precipitation. The purified DNA and input genomic DNA were analyzed by real time PCR. The results of mH2A1 binding are normalized against input DNA. The following primers were used for ChIP: −1703 kb primers (PCR product length, 91 bp): H2pro1703F, 5′-GGTTGCCAC TCCA-GACTTGTG-3′; H2pro1703R, 5′-TCTCTCACCTCCTCT- TTCC-3′; −1215 kb primers (PCR product length, 400 bp): H2pro1215F, 5′-GGCAAAGAGCAAGAATGCTCC-3′; H2pro1215R, 5′-CACACTTTTCTCGGAGAATCC-3′; −1142 kb primers (PCR product length, 245 bp): H2pro1142F, 5′-AGGCCAGAAGTACTTGAACC-3′; H2pro1142R, 5′-AGGGGGATGTGTTGTTTACC-3′; −838 kb primer (PCR product length, 327 bp): Her2pro838F, 5′-TAAGCAGGCTTATGAAGGC-3′; Her2pro838R, 5′-CTGTCTCAAGTGATTCC-3′; −226 kb primer (PCR product length, 390 bp): Her2pro226F, 5′-GACAGGGTTTCACCGTGTTAGC-3′; Her2pro226R, 5′-GGGCAACAAGAGCAAAAGTTCG-3′; −52 kb primer set (PCR product length, 384 bp): H2pro521F, 5′-AGGATACAAGTGCTCATCAAGG-3′; H2pro521R, 5′-CCCTTGCCTTCTATTAGCCTGC-3′; −226 kb primer (PCR product length, 188 bp): H2pro64F, 5′-AGTCTCACTCTGTCTCC-3′; H2pro64R, 5′-TCTCTGCTACAACGGTGAA-3′, and −5 kb primer (PCR product length, 384 bp): H2pro5KF, 5′-AGGATAAAGTGCTGTCATCAAGG-3′; 5′-CTC- TACCTACTTACCAGCC-3′.

RESULTS

HER-2 Interacts with the Atypical Histone Macrohistone 2A1—To identify HER-2 interacting proteins, a TAP-ERBB2 construct was transiently expressed in HEK 293 cells, which express endogenous HER-2. The TAP-HER-2 fusion protein utilized expressed tandem N-terminal streptavidin and calmodulin binding peptide sequences allowing for affinity purification. A number of proteins copurified with TAP-HER-2 protein (Fig. 1A). In particular, one protein of ~42 kilodaltons repeatedly copurified with TAP-HER-2 following extensive washing (Fig. 1B) and was identified as mH2A1 by tandem mass spectrometry.

HER-2 interacts with a number of proteins such as phosphatidylinositol 3-kinase, GRB-2, and SHC via tyrosine phosphorylation sites on its cytoplasmic domain. To determine whether the interaction between mH2A1 and HER-2 was dependent on HER-2 phosphorylation, two HER-2 antibodies were used to co-immunoprecipitate HER-2 and mH2A1. One HER-2 antibody, which does not recognize the tyrosine phos-
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The ability of mH2A1.2 to increase ERBB2 mRNA levels in phorylated form of HER-2, was clearly able to co-immunoprecipitate mH2A1 with HER-2 (Fig. 1C). A second and biotinated HER-2 antibody, which recognizes the phosphorylated form of HER-2, was capable of co-immunoprecipitating mH2A1 as well (Fig. 1D). These data suggest that HER-2 interacts well with mH2A1 regardless of the tyrosine phosphorylation status of HER-2.

SKBR-3 and SKOV-3 cancer cell lines are cancer cell lines with ERBB2 gene duplication and express high levels of HER-2. To determine whether HER-2 and mH2A1 expression were related, SKBR-3 and SKOV-3 cells were treated with antagonistic HER-2 mAb for various times, and then mH2A1 protein levels were determined by immunoblotting. For both cell lines, 8 h of treatment with anti-HER-2 mAb diminished the levels of mH2A1 and HER-2 protein expression (Fig. 1E), suggesting that HER-2 plays a role in mH2A1 expression. The diminished levels of HER-2 may have been due to the decreased levels of mH2A1.

To determine whether HER-2 affects mH2A1 levels in cancer cells that express HER-2 but lack ERBB2 gene duplication, Caco-2 cells were treated with anti-HER-2 mAb. We showed previously that anti-HER-2 mAb treatment of Caco-2 cells increased phospho-HER-2 levels but inhibited HER-2 tyrosine kinase activity (25). Treatment of Caco-2 cells with anti-HER-2 mAb increased phospho-HER-2 levels but diminished mH2A1 protein and mRNA levels (Fig. 1, F and G) demonstrating a dependence of mH2A1 levels on HER-2 tyrosine kinase activity.

mH2A1.2, but Not mH2A1.1, Drives Tumorigenicity in Cancer Cells—The mH2A1 antibody used in this study preferentially binds to mH2A1.2 over mH2A1.1 but nevertheless binds to the two isoforms. Only one previous study examined the difference between mH2A1.1 and mH2A1.2 in terms of tumorigenicity and found that mH2A1.1 expression was a prognostic factor for lung cancer recurrence (26).

To further evaluate the potential roles of the mH2A1 isoforms in tumorigenicity, SKBR-3 breast cancer cells were stably transfected with full-length and wild-type human mH2A1.1 and mH2A1.2 constructs. In soft agar colony assays, cells stably transfected with mH2A1.2, but not mH2A1.1, produced large multicellular colonies after 1 week (Fig. 2A), demonstrating a much greater ability of mH2A1.2 to drive anchorage-independent proliferation. When the same cell lines were injected subcutaneously in athymic mice, the cells stably transfected with mH2A1.2, but not mH2A1.1, generated tumors significantly larger than the controls (Fig. 2B). SKBR-3 cells stably transfected with mH2A1.2 proliferated much more rapidly than controls over 8 days (Fig. 2C), and treatment of those cells with anti-HER-2 mAb inhibited their proliferation to the level of the control cells (untreated) (Fig. 2C). Further evaluation of the proliferation data shows that anti-HER-2 mAb inhibited proliferation of SKBR-3 cells transfected with mH2A1.2 much more than SKBR-3 control cells (Fig. 2D). Thus, the results suggest that overexpression of mH2A1.2 induced cell proliferation through increased HER-2 activity, and mH2A1.2, but not mH2A1.1, increased the proliferation and tumorigenicity of HER-2-overexpressing cancer cells.

mH2A1.2, but Not mH2A1.1, Interacts with HER-2—Because the mH2A1 antibody used could not distinguish the two mH2A1 isoforms, MYC epitope-tagged mH2A1.1 and mH2A1.2 constructs were transiently transfected into HEK 293 cells, which express low levels of mH2A1 but express HER-2. Only mH2A1.2-MYC co-immunoprecipitated endogenous HER-2 (Fig. 3A), indicating that mH2A1.2, but not mH2A1.1, interacts with HER-2. To determine whether mH2A1.2 affected HER-2 expression in cancer cells, SKOV-3 and SKBR-3 cells were stably transfected with full-length human mH2A1.2 constructs, which increased HER-2 protein expression in both cell lines (Fig. 3B). SKOV-3 and SKBR-3 cells stably transfected with mH2A1.2 demonstrated 2.7- and 1.6-fold-increased HER-2 mRNA levels, respectively, by quantitative RT-PCR (Fig. 3C), suggesting that mH2A1.2 overexpression induced ERBB2 transcription.

mH2A1 and HER-2 Colocalize in the Nuclei of Cancer Cells—The ability of mH2A1.2 to increase ERBB2 mRNA levels in

FIGURE 3. mH2A1.2, but not mH2A1.1, interacts with HER-2. A, HEK 293 cells were transiently transfected with vector alone or MYC epitope-tagged mH2A1.1 and mH2A1.2 expression constructs for 48 h. The cells were lysed and normalized for total protein concentration. A Western (W) blot of HER-2 shows that all cell lines expressed endogenous HER-2 (top panel). MYC-tagged proteins were immunoprecipitated (IP) with MYC antibodies, and the proteins were resolved by SDS-PAGE. Western blots were performed for HER-2 and MYC on the immunoprecipitates to detect co-immunoprecipitation of HER-2 with MYC-tagged proteins. The results shown are typical of two separate experiments. B, SKBR-3 and SKOV-3 cells that were stably transfected with vector alone or MYC-mH2A1.2 were lysed. After normalization for total protein concentration, the lysates were used for Western blots. The results shown are typical of at least four separate experiments. C, quantitative RT-PCR results for ERBB2 mRNA purified from SKBR-3 and SKOV-3 cells that were stably transfected with mH2A1.2. The results are expressed as the means of the -fold differences between ERBB2 and GAPDH mRNA levels (± S.E.) for three experiments. * p < 0.05.
cancer cells suggested an interaction of mH2A1.2 and HER-2 within the nucleus. HER-2 was found in the cell membrane and nucleus by immunofluorescence localization in SKOV-3 cells (Fig. 4A). mH2A1 was found in the cytoplasm and nuclei of SKOV-3 cells (Fig. 4B), and colocalization of mH2A1 and HER-2 occurred in the nuclei of SKOV-3 cells (Fig. 4B). Phospho-HER-2 was predominantly found in the nuclei of SKOV-3 cells (Fig. 4C). SKBR-3 cells showed similar immunolocalization of HER-2 and mH2A1 (not shown).

Cell fractionation studies showed that phospho-HER-2 co-immunoprecipitated with mH2A1 in the cytoplasmic and nuclear fractions of SKBR-3 and SKOV-3 cells (Fig. 4D).

*mH2A1.2 Increases HER-2 Expression through a Nuclear Mechanism*—Because mH2A1 was clearly found in the cytoplasm and nuclei of SKBR-3 and SKOV-3 cells, it was important to determine whether mH2A1.2 increased HER-2 expression through transcriptional or post-transcriptional means. A fusion protein that expressed the SV40 large T antigen NLS at the C-terminal end of mH2A1.2 was constructed to force its expression in the nucleus. SKOV-3 cells that were stably transfected with mH2A1.2-NLS (MYC epitope-tagged) expressed the MYC-tagged fusion protein in the nuclear, but not cytoplasmic, fraction of the cells (Fig. 5A). Although stable expression of mH2A1.2 resulted in a 2.1-fold increase in ERBB2 mRNA levels, expression of mH2A1.2-NLS caused a 2.5-fold increase in ERBB2 mRNA levels in SKOV-3 cells (Fig. 5B). When the same cell lines were transiently transfected with an ERBB2-luciferase reporter construct, stable expression of mH2A1.2 led to an ~4-fold increase in ERBB2 promoter activity, whereas mH2A1.2-NLS resulted in an ~5-fold increase in ERBB2 promoter activity (Fig. 5C). Because the mH2A1.2-NLS fusion protein was only expressed in the nuclear fraction of SKOV-3 cells and caused increases in ERBB2 mRNA and ERBB2 promoter activity similar to that of mH2A1.2 alone, it is likely that
mH2A1.2 increased HER-2 expression primarily through increased transcription. Xenografting of the transfected cells in mice showed that mH2A1.2-NLS cells formed significantly larger tumors than mH2A1.2-transfected cells (Fig. 5D). These data suggest that the predominant effect of mH2A1.2 on HER-2 expression and tumorigenicity is due to its nuclear activity.

mH2A1.2 Is Enriched within the ERBB2 Promoter in Cancer Cells—Co-transfection of an ERBB2 promoter-(firefly) luciferase construct and a full-length mH2A1.2 construct in SKOV-3 cells resulted in a large increase in ERBB2 promoter activity (Fig. 6A). Co-transfection of kinase-dead HER-2 with mH2A1.2 abrogated mH2A1.2-induced ERBB2 promoter activation in SKOV-3 cells, suggesting that HER-2 kinase activity is required for mH2A1.2-induced ERBB2 expression (Fig. 6A). These results suggested that macroH2A1.2 overexpression increased the transcription of ERBB2 in a HER-2 kinase-dependent fashion.

mH2A1.2 is best known as a transcriptional repressor particularly with regard to the inactivated X chromosome (27). However, a recent study demonstrated that mH2A1 occupancy can protect a number of autosomal genes from silencing and thus can positively regulate gene transcription (28). Thus, chromatin immunoprecipitation was used to determine whether mH2A1.2 overexpression in cancer cells led to increased or decreased occupancy of the ERBB2 promoter. mH2A1.2 occupancy of regions of the ERBB2 promoter encompassing a region up to 5 kb upstream of the translational start site was compared between control (vector only) and mH2A1.2-transfected SKOV-3 cells. At five of eight positions interrogated along the 5-kb stretch of the ERBB2 promoter, mH2A1.2 expression was significantly enriched in SKOV-3 cells stably transfected with mH2A1.2 compared with the control cells (Fig. 6B). At two of eight positions, there was a non-significant trend for increased mH2A1.2 enrichment in SKOV-3 cells stably transfected with mH2A1.2 compared with the control cells (Fig. 6B). Only one position (~1703 kb) showed less mH2A1.2 occupancy of the HER-2 promoter in SKOV-3 cells stably transfected with mH2A1.2 compared with the control cells (Fig. 6B). These results suggested that mH2A1.2 overexpression in cancer cells is accompanied by a net increase in mH2A1.2 occupancy of the ERBB2 promoter, which corresponds with an increase in ERBB2 transcription in the same cells.

The Macro Domain of mH2A1.2 Mediates Its Interaction with HER-2—mH2A1 is an atypical histone that consists structurally of an H2A-like domain (70% amino acid sequence identity with canonical histone 2A) followed by a linker/leucine zipper region that connects to a large macro domain (29). The macro domain is a highly conserved motif in prokaryotes and eukaryotes that mediates interactions with a variety of proteins and NAD+ metabolites (30). To determine which portion of mH2A1.2 was required for interaction with HER-2, HEK 293 cells were transiently transfected with epitope-tagged mH2A1.2 constructs (Fig. 7A). To overcome overlapping of mH2A1.2 protein with the heavy immunoglobulin chain protein in Western blots of co-immunoprecipitants of mH2A1.2, various epitope tags were used to generate fusion proteins of full-length and mutated mH2A1.2. The macro domain and full-length mH2A1.2 constructs were expressed as HALO epitope-tagged proteins, which have predicted molecular weights much larger than immunoglobulin heavy chain. HALO fusion proteins can be purified through a biotinylated and cell-permeable ligand that binds covalently and specifically to the macro domain. ERBB2 co-purified with the HALO-macroH2A1.2 fusion and HALO-macro domain fusion proteins using streptavidin beads (Fig. 7, B–D). The H2A-like domain was expressed as a V5 epitope-tagged fusion protein. The H2A-V5 fusion protein failed to co-immunoprecipitate HER-2 (Fig. 7E). These results showed that HER-2 interacts with macroH2A1.2 through its macro domain.

The -EIS- Sequence in the Macro Domain of mH2A1.2 Mediates Its Interaction with HER-2—A comparison of the amino acid sequences of mH2A1.1 and mH2A1.2 shows divergence predominantly in a stretch of amino acids in the proximal macro domain that immediately follows the conserved leucine zipper domain (Fig. 8A). The most conspicuous difference between the crystal structures of the macro domains of mH2A1.1 and mH2A1.2 is the presence of a three-amino acid (-EIS-) insertion unique to mH2A1.2 (Fig. 8, B and C). Compar-
Comparison of the crystal structures of these unconserved regions of mH2A1.1 and mH2A1.2 macro domains reveals a groovelike feature and the -EIS- sequence of mH2A1.2 that projects prominently across the groovelike feature (Fig. 8, D and E).

To study the unique features of the mH2A1.2 macro domain, V5 epitope-tagged mutant constructs of mH2A1.2 were generated and stably expressed in SKOV-3 cells (Fig. 8F). Deletion of a 31-amino acid sequence, encompassing the leucine zipper region and proximal macro domain of mH2A1.2 (Δ31) containing the -EIS- sequence, prevented its interaction with HER-2 (Fig. 8G). We hypothesized that the relatively exposed and polar amino acids within the -EIS- sequence in mH2A1.2 may be required for interaction with HER-2. Expression of a full-length mH2A1.2 construct (E202G/S204A) in which the glutamate and serine residues in the -EIS- sequence were mutated to glycine and alanine, respectively, prevented its interaction with HER-2 (Fig. 8G). Both full-length mH2A1.1 and the H2A-like domain of mH2A1.2 failed to co-immunoprecipitate with HER-2 (Fig. 8G). These results show that the -EIS- insertion sequence unique to mH2A1.2 is important for the interaction of mH2A1.2 with HER-2.

Transient expression of the macro domain of mH2A1.2 in HEK 293 cells, which express HER-2, was sufficient to increase ERBB2 promoter activity similar to that of full-length mH2A1.2 (Fig. 8H), suggesting that the macro domain of mH2A1.2 was sufficient for mH2A1.2-induced ERBB2 transcription. Tran-
sient expression of mH2A1.1 or the H2A-like domain failed to increase ERBB2 promoter activity compared with the control (Fig. 8H). Deletion of a 31-amino acid region containing the -EIS- insertion (Δ31) or point mutation of the -EIS- domain to -GIA- (E202G/S204A) abrogated mH2A1.2-induced ERBB2 promoter activity (Fig. 8H), suggesting that the -EIS- domain of mH2A1.2 is required for mH2A1.2-induced ERBB2 activation.

Expression of the various proteins was similar (not shown).

The H2A-like Domain of mH2A1.2 Exerts a Dominant-Negative Effect on HER-2 Overexpression—The H2A-like domain of mH2A1.2 shares 70% of its amino acid sequence with the canonical histone H2A. Stable expression of the H2A-like domain of mH2A1.2 in SKOV-3 cells (Fig. 9, A–C) led to a striking decrease in HER-2 protein (Fig. 9A) as well as endogenous mH2A1.2 (Fig. 9B), showing that loss of endogenous mH2A1.2 is required for mH2A1.2-induced ERBB2 expression. Expression of the various proteins was similar (not shown).

The H2A-like Domain of mH2A1.2 Exerts a Dominant-Negative Effect on HER-2 Overexpression—The H2A-like domain of mH2A1.2 shares 70% of its amino acid sequence with the canonical histone H2A. Stable expression of the H2A-like domain of mH2A1.2 in SKOV-3 cells (Fig. 9, A–C) led to a striking decrease in HER-2 protein (Fig. 9A) as well as endogenous mH2A1.2 (Fig. 9B), showing that loss of endogenous mH2A1.2 is required for HER-2 protein expression in cancer cells. SKOV-3 cells stably expressing the H2A-like domain demonstrated a significant decrease in ERBB2 mRNA levels by quantitative RT-PCR, suggesting that the H2A-like domain exerted a dominant-negative effect on endogenous mH2A1 (Fig. 9D), which in turn inhibited HER-2 expression.

Functionally, stable transfection of the macro domain and full-length mH2A1.2 constructs in SKOV-3 cells significantly increased cell proliferation compared with control cells (Fig. 9C), whereas stable expression of the H2A-like domain mutant construct inhibited cell proliferation compared with the control cells (Fig. 9C). These results showed that the macro domain of mH2A1.2 is sufficient for mH2A1.2-induced HER-2 expression and cell proliferation.

DISCUSSION

mH2A1 is an atypical histone whose characteristic feature is its large macro domain. Macro containing proteins are highly evolutionarily conserved and mediate interactions with various molecules (30). In normal cells, mH2A1 is best known as a transcriptional repressor and for its role in X chromosome inactivation (27). Human cells express two isoforms of mH2A1, mH2A1.1 and mH2A1.2, generated through alternative splicing of the H2AFY gene. Significant differences between mH2A1.1 and mH2A1.2 have been noted previously. 1) mH2A1.1 binds O-Ac-ADP-ribose (31). 2) mH2A1.2 is enriched in the heterochromatin of senescent cells (32). 3) mH2A1.2 is monoubiquitinated (32). 4) mH2A1.1 appears to be expressed more in differentiated cells and is down-regulated in certain cancers (26, 33), suggesting that mH2A1.1 may possess tumor suppressor functions.
Our study revealed novel roles of mH2A1.2 in cancer cells. We found that mH2A1.2, but not mH2A1.1, overexpression in cancer cells increased cell proliferation and tumorigenicity in concert with HER-2. We showed that the macro domain of mH2A1.2, but not mH2A1.1, interacted with HER-2 and that this interaction was required for mH2A1.2-induced ERBB2 transcription. As our study was limited to cancer cells overexpressing HER-2 and mH2A1, we do not know whether mH2A1.2 can interact with HER-2 or other receptor tyrosine kinases in normal cells expressing much lower levels of these proteins. However, the observation of c-erbB receptors in the nuclei of normal cells suggests that this interaction may be possible.
The major region of divergence in the amino acid sequences of mH2A1.1 and mH2A1.2 occurs within the N-terminal portion of their macro domains, and it is this region that was shown to be required for interaction with HER-2. In particular, the unique -EIS- insertion within the macro domain of mH2A1.2 was shown to be critical for the interaction between mH2A1.2 and HER-2 and mH2A1.2-induced ERBB2 transcription. The -EIS- sequence of mH2A1.2 protrudes into a groovelike region of the protein. The analogous groovelike region in mH2A1.1 provides a high affinity binding site for the SirT1 metabolite O-acetyl-ADP-ribose, which has been implicated in transcriptional silencing (31). Asp-203 within the macro domain of mH2A1.1 is very important for the interaction between mH2A1.1 and O-acetyl-ADP-ribose and corresponds with the analogous part of mH2A1.2 that contains the -EIS- sequence (31). On the other hand, mH2A1.2 cannot bind nucleotides with high affinity, and this has been attributed in part to the -EIS- insertion that protrudes into the corresponding O-acetyl-ADP-ribose binding site of mH2A1.1 (31).

The expression of the macro domain of mH2A1.2 in cancer cells was able to induce ERBB2 promoter activity and cell proliferation to a degree similar to that of full-length mH2A1.2. On the other hand, expression of only the H2A-like domain of mH2A1.2 in cancer cells inhibited both HER-2 and mH2A1 expression. These results suggest that the macro domain alone can increase HER-2 interaction with endogenous mH2A1.2 and HER-2 drives expression of mH2A1.2, which then cooperates with HER-2 to increase HER-2 overexpression in cancer cells. In support of this mechanism was the finding that inhibition of HER-2 in SKBR-3, SKOV-3, and Caco-2 cancer cells caused a decrease in mH2A1 expression.

Our findings that stable overexpression of mH2A1.2 in cancer cells increased ERBB2 promoter occupancy, ERBB2 promoter activity, and ERBB2 mRNA levels suggested that mH2A1.2 may be activating rather than repressing ERBB2 transcription. Although the majority of studies of mH2A1 demonstrate its role in transcriptional repression, a recent study (28) using IMR90 lung fibroblasts and MCF-7 breast cancer cells revealed exceptions. Although most autosomal genes marked by mH2A1 were transcriptionally repressed, a subset of those genes was found to be transcriptionally active and thus appeared to be protected from mH2A1-mediated transcriptional repression (28). Furthermore, genes (ASCL1, CST5, SOCS2, TFF1, and TMPRSS3) that demonstrated mH2A1 binding downstream of the transcriptional start site and induction by serum starvation demonstrated decreased expression following RNAi-mediated inhibition of mH2A1, suggesting a role for mH2A1 in transcriptional activation (28). Importantly, serum starvation induced expression of these genes but did not

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**TABLE 1**

Co-overexpression of HER-2 and macroH2A1 in human cancer microarray studies

Several microarray studies of breast, ovarian, and colorectal cancer mRNA samples in the Oncomine database were analyzed to determine what percentage of cancers overexpressing HER-2 or macroH2A1 mRNA compared with normal tissue also overexpressed macroH2A1 or HER-2 mRNA.

| Study              | Cancer type       | HER-2-overexpressing cancers with macroH2A1 overexpression | MacroH2A1-overexpressing cancers with HER-2 overexpression | Total number of cases |
|--------------------|-------------------|------------------------------------------------------------|----------------------------------------------------------|-----------------------|
| Richardson et al.  | Breast            | 100%                                                       | 65%                                                      | 40                    |
| Sorlie et al.      | Breast            | 93.8%                                                      | 62.5%                                                    | 78                    |
| Perou and co-workers | Breast, ductal    | 98.0%                                                      | 93.3%                                                    | 54                    |
| Lu et al.          | Ovarian, serous   | 100%                                                       | 94.7%                                                    | 19                    |
| Hendrix et al.     | Ovarian, endometrioid | 97.3%                                                  | 100%                                                     | 37                    |
| Graudens et al.    | Colorectal        | 100%                                                       | 14.3                                                     | 18                    |

* p < 0.05 for cancers co-overexpressing macroH2A1 and HER-2 compared with cancers that did not.
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alter occupany of mH2A1 upstream of or at the translational start site or in the transcribed regions (28), suggesting the involvement of transcriptional co-activators.

It remains possible that mH2A1.2 may modulate ERBB2 transcription indirectly through the regulation of transcriptional factors such as PEA-3, which binds to and represses the ERBB2 promoter (35); however, we did not find significant differences in PEA3 mRNA expression between cancer cells overexpressing mH2A1.2 and control cells. The novel finding of a functional and cooperative interaction between a protooncogene and atypical histone may have strong implications for oncogenesis. HER-2 is commonly overexpressed in a variety of cancers (1–6) and has been readily demonstrated in the nuclei of cancer cells in other studies (23, 24) and the present study. HER-2-overexpressing breast cancers have a distinctive mRNA expression profile and clinical behavior compared with breast cancers lacking HER-2 overexpression (36), and a review of human tumor microarrays demonstrated a high correlation between mH2A1 and HER-2 mRNA expression (37) and the present study. HER-2-overexpressing breast cancers expressed in a variety of cancers (1–6) and has been readily demonstrated in the cytoplasm (37) and nuclei (23, 24) of human cancer cells in situ. Although HER-2-directed antibodies might be sufficient to inhibit HER-2 signaling and stimulate antibody-dependent cell-mediated cytotoxicity, this strategy may be insufficient to fully inhibit HER-2/mH2A1.2 interactions within the cell. Expression of mutant mH2A1.2 proteins no longer capable of interacting with HER-2 inhibited cancer cell proliferation in the present study, suggesting that inhibition of mH2A1.2/HER-2 interactions may be a successful therapeutic strategy in cancers overexpressing these proteins.

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