YY1 modulates taxane response in epithelial ovarian cancer

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Statement of Clinical Relevance

The results of this study show that a high YY1 gene signature (characterized by coordinate elevated expression of transcription factor *YY1* and putative YY1 target genes) within serous epithelial ovarian cancers is associated with enhanced response to taxane-based chemotherapy and improved survival. If confirmed in a prospective study, these results have important implications for the potential future use of individualized therapy in treating patients with ovarian cancer. Identification of the *YY1* gene signature profile within a tumor prior to initiation of chemotherapy may provide valuable information about the anticipated response of these tumors to taxane-based drugs, leading to better informed decisions regarding chemotherapeutic choice.

ABSTRACT

Purpose. Survival of ovarian cancer patients is largely dictated by their response to chemotherapy, which depends on underlying molecular features of the malignancy. We previously identified *YIN YANG 1* (*YY1*) as a gene whose expression is positively correlated with ovarian cancer survival. Herein we investigated the mechanistic basis of this association.

Experimental design. Epigenetic and genetic characteristics of *YY1* in serous epithelial ovarian cancer (SEOC) were analyzed along with YY1 mRNA and protein. Patterns of gene expression in primary SEOC and in the NCI60 database were investigated using computational methods. YY1 function and modulation of chemotherapeutic response *in vitro* was studied using siRNA knockdown.

Results. Microarray analysis showed strong positive correlation between expression of *YY1* and genes with YY1 and transcription factor E2F binding motifs in SEOC and in the NCI60 cancer cell lines. Clustering of microarray data for these genes revealed that high YY1/E2F3 activity positively correlates with survival of patients treated with the microtubule stabilizing drug paclitaxel. Increased sensitivity to taxanes, but not to DNA crosslinking platinum agents, was also characteristic of NCI60 cancer cell lines with a high YY1/E2F signature. *YY1* knockdown in ovarian cancer cell lines results in inhibition of anchorage-independent growth, motility and proliferation, but also increases resistance to taxanes, with no effect on cisplatin sensitivity.

Conclusions. These results, together with the prior demonstration of augmentation of microtubule-related genes by E2F3, suggest that enhanced taxane sensitivity in tumors with high YY1/E2F activity may be mediated by modulation of putative target genes with microtubule function.

INTRODUCTION

Epithelial ovarian cancer is the leading cause of death from gynecologic malignancies in the United States, with 21,650 new cases and 15,520 deaths expected this year alone (http://www.cancer.gov). Most cases are metastatic when diagnosed and are treated with systemic chemotherapy consisting of a combination of taxane, a microtubule stabilizing agent, and platinum drugs that form DNA and protein crosslinks. The majority of women with advanced stage ovarian cancer succumb within five years due to recurrence of chemoresistant disease. One of the major obstacles to improving patient survival is the lack of understanding regarding the molecular characteristics of ovarian malignancies that contribute to chemotherapeutic response. Delineating these features may provide the means to improve patient prognosis in the future by allowing treatment strategies to take into account the likelihood that a given malignancy will respond to treatment favorably based on its underlying molecular phenotype.

Previously we reported that elevated *YIN YANG 1* (*YY1*) expression is positively correlated with enhanced survival of ovarian cancer patients based on gene expression microarray analysis (1). This finding was confirmed using an independent ovarian cancer microarray gene expression dataset (2), supporting that YY1 plays a significant role in ovarian cancer prognosis. We therefore hypothesized that elevated YY1 expression is functionally involved in positively influencing the response to chemotherapeutic agents for women diagnosed with epithelial ovarian cancer. *YY1* encodes a GLI-Krüppel type zinc finger protein that is ubiquitously expressed and conserved among vertebrates (3-7), with functional homologs present in Drosophila (8). YY1 protein has divergent cellular functions, including the ability to activate and repress gene transcription, modulate the function of other proteins, and affect chromatin structure (9). *YY1* transcription and function is also tied to progression of the cell cycle (10-13), implicating a possible role in carcinogenesis (9, 14). YY1 was previously shown to

function synergistically with E2F2 and E2F3 to activate target gene transcription in a molecular complex bridged by the Ring1- and YY1-binding protein (RYBP) (12). Such interactions facilitate the transcriptional activation of target genes that are involved in cell cycle progression and mitosis (15).

Based on analysis of two independent sets of microarray data, including our serous epithelial ovarian cancer data and the NCI60 dataset comprising 59 cell lines of multiple cancer types, we show herein that YY1 in association with E2F upregulates target genes having YY1 and E2F binding motifs. We further sought to understand the reasons underlying the differences in survival of ovarian cancer patients with respect to expression of *YY1*. We found that high YY1 activity correlates with sensitivity to taxanes and better prognosis of ovarian cancer patients. Furthermore, we uncovered an association between YY1 and E2F that regulates transcription of genes encoding proteins involved in microtubule-associated functions.

MATERIALS AND METHODS

Tissues and cell lines.

All tissues were obtained with consent and used with approval from the Duke University Institutional Review Board. Human conceptal tissues were provided by the NIH-supported Laboratory of Human Embryology at the University of Washington, Seattle. Malignant and normal ovarian tissues and lymphocytes were provided by the Gynecologic Oncology Tumor Bank at Duke University Medical Center. Ovarian cancer cell lines were cultured in RPMI 1640 media (Invitrogen Co. Carlsbad, CA) with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin solution (Invitrogen) in a 37ºC humidified incubator with 5% atmospheric CO_2.

Microarray datasets.

Microarray data (Affymetrix U133A genechips) was generated and normalized as previously described (1). This microarray data includes 12 serous borderline tumors, 12 early stage (stage I/II) serous epithelial ovarian cancers, 24 advanced stage (III/IV) serous ovarian cancers (living ≥7y post diagnosis), 33 advanced stage serous ovarian cancers (living ≤3y post-diagnosis) and 7 advanced stage recurrent serous epithelial ovarian cancers. The clinicopathological features of this patient population were described previously (1). We used microarray gene expression data from an additional 79 advanced serous epithelial ovarian cancers for external validation. This dataset is derived from previously published data (16) by excluding samples overlapping with the above dataset (1). NCI60 data from Affymetrix U95Av2 microarrays was also used (http://www.dtp.nci.nih.gov/).

Transcription factor binding motif analysis.

We retrieved gene promoter sequences, as annotated by RefSeq, from -1,200 bases upstream to 200 bases downstream of the transcription start site from the hg17 assembly of the

human genome from the UC Santa Cruz Genome Browser (17, 18). We mapped those sequences to the probes on the Affymetrix microarrays via the common Gene IDs provided by RefSeq and NetAffx. We then scanned each of the promoter sequences for binding motifs using TRANSFAC version 8.2 (19). We discarded the hits whose scores did not exceed the cutoffs recommended in the minSUM_good74 file provided in the TRANSFAC database. Based on the probe annotation for RefSeq IDs, a TRANSFAC annotation file was generated for the Affymetrix probes.

Correlation to *YY1*.

Affymetrix U133A *YY1* probes with Log_2 expression values >7 in at least one sample were used for ovarian tumor dataset analysis following REDuction of Invariant probes (REDI; kindly performed by Expression Analysis, Inc., Durham, NC; Table SI). REDI allowed us to exclude probes that were defined as "not responsive" or " invariant", although this did not cause a significant difference in results. *YY1* probes used for the calculation of correlation coefficients included 200047_s_at and 201901_s_at and expression values from these probes were averaged. Probe 891_at in the U95Av2 probe set, annotated as "best match" with either of the above two probes, was used in both the NCI60 dataset and the external validation dataset. Pearson product-moment correlation coefficients to *YY1* were calculated for all the probes using the correlation (CORREL) function in EXCEL for each dataset.

Clustering and heat maps.

Clustering was performed using Cluster 3.0, available from the website, http://rana.lbl.gov/EisenSoftware.htm. Genes for clustering were selected as described above. Prior to clustering, gene expression was median-centered and normalized in Cluster 3.0. Heat maps for expression microarray data were made with Java TreeView (http://jtreeview.sourceforge.net/). Heat maps for correlation coefficients and E2F3 signature

probability data were made using the statistical computing and graphics environment, R (http://www.rproject.org/). We filtered genes with low expression in the majority of tumors by using $\log_2$ transformed expression values and retained those with high expression (≥ 7) in one or more tumors (20). Of 22,215 probes on the Affymetrix U133A genechip, approximately 13,000 met this criterion. The top 250 *YY1* positively correlated genes that contain both YY1 and E2F binding motifs according to TRANSFAC analysis (V$YY1_Q6 and V$E2F1_Q3, respectively) were selected for clustering after median centered normalization using Cluster. For NCI60 U95Av2 dataset, top 150 probes were selected and clustering was conducted in the same way.

E2F3 signature probability.

The E2F3 overexpression gene signature was previously developed using microarray data from recombinant adenovirus-infected primary mammary epithelial cells (16). This microarray dataset was also used to analyze transcriptional regulation of microtubule related genes by E2F3.

Gene Set Enrichment Analysis (GSEA).

GSEA was performed using the GSEA software available from http://www.broad.mit.edu/gsea/ (21). The Affymetrix U133 probe lists having Gene Ontology designations of “microtubule” (GO#5874) or “microtubule associated complex” (GO#5875) were generated from the annotation files available from the Affymetrix website (http://www.affymetrix.com/). Enrichment of these gene lists was examined between ten control and nine E2F3 overexpressing samples (16). False Discovery Rates (q) < 0.25 were considered biologically relevant.

***YY1* knockdown.**

Cells from the BG1 and HEY ovarian cancer cell lines were seeded into a 24-well plate at a density of $1x10^5$ cells/well followed by transfection with 5 nM of either control non-silencing siRNA oligos or YY1-specific siRNA oligos (Qiagen; Valencia, CA) using HiPerfect reagent (Qiagen) according to the manufacturer's recommendations. *YY1*-specific siRNA oligo sequences were as follows: siRNA target 1 sequence: GAC GAC GAC TAC ATT GAA CAA; siRNA 1 sense oligo: r(CGA CGA CUA CAU UGA ACA A)dTdT; siRNA 1 antisense oligo: r(UUG UUC AAU GUA GUC GUC G)dTdC; siRNA 2 target sequence: AAC CTG AAA TCT CAC ATC TTA; siRNA 2 sense oligo: r(CCU GAA AUC UCA CAU CUU A)dTdT; siRNA 2 antisense oligo: r(TAA GAT GTG AGA TTT CAG G)dTdG. The cells were trypsinized 24 hours post-transfection and seeded for further analysis as described below. The remaining cells were seeded into a 6-well plate and cultured for an additional 48 hours for determination of knockdown efficiency.

Reverse transcription.

YY1, *CDC6*, and *MCM5* transcript levels were determined using quantitative real time PCR (TaqMan Assays on Demand, Assay IDs: Hs00231533_m1, Hs00154374_m1, ,Hs00198823_m1, respectively; Applied Biosystems) after generation of oligo dT primed cDNA using Superscript II reverse transcriptase (Invitrogen). Initial experiments showed that siRNA-mediated knockdown of *YY1* also variably affected the expression of *ß2-Microglobulin*, intended as a control for input RNA normalization (TaqMan Assays on Demand, Assay ID Hs00187842_m1). All reverse transcription reactions were performed with one µg of total RNA as input measured on a Nanodrop 1000 spectrophotometer (Thermo Scientific; Wilmington, DE). We therefore directly utilized cycle threshold values for all assessments of YY1 mRNA levels in real time RT-PCR experiments, which were performed at least in duplicate.

Western blotting.

Hey and BG1 cells were transiently transfected in two independent experiments with *YY1* siRNA or control non-silencing siRNA as described above and incubated for 48 or 72 hours prior to harvesting. Whole cell lysates were prepared using Biosource NP40 cell lysis buffer (Invitrogen). Proteins were resolved by SDS-PAGE (4-15%) and transferred to a nitrocellulose membrane (Biorad; Hercules, CA). A 1:200 dilution of mouse anti-human YY1 antibody (H-10) (sc-7341, Santa Cruz Biotechnology; Santa Cruz, CA) was used followed by detection with a 1:3000 dilution of secondary antibody (Biorad) and the Enhanced Chemiluminescence system (Amersham Biosciences; Piscataway, NJ). Mouse anti-GAPDH antibody was used as an internal loading control.

Cell proliferation assays.

10^3 BG1 or HEY ovarian cancer cells were seeded into individual wells of a 96-well plate containing 100 µl of culture medium followed by transfection with control or *YY1*-specific siRNA oligos in quadruplicate. 96 hours post-transfection, cell proliferation was measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay according to the manufacturer's recommendations (Promega; Madison, WI).

Anchorage-independent growth assays.

10^3 BG1 or HEY ovarian cancer cells were seeded into individual wells of a 96-well plate containing 0.5% agar / RPMI 1640 followed by transfection with control or *YY1*-specific siRNA oligos in quadruplicate. The cells were cultured for 7-10 days prior to counting colonies >100mm in diameter.

Wound healing assays.

BG1 and HEY ovarian cancer cells were transfected with control and *YY1*-specific siRNA oligos in a 24-well plate as described above. 24 hours post-transfection, the cells from two wells were combined and seeded into one well of a 6-well plate. The cells reached near confluence (>90%) 72 hours after transfection. 'Wounds' were introduced through the cell monolayer at 72 hours post-transfection using a P-1000 pipet tip. The cells were gently rinsed with PBS and cultured in RPMI 1640 medium containing 10% FBS. Micrographs were taken at time 0 and at 5, 20, and 28 hours post-wounding using the 10× objective of an inverted phase contrast microscope. The line tool in Canvas 9 (ACDSee Systems; Miami, FL) was used on the digital image to measure the distance between the facing edges of the expanding cells within the gap at five roughly equidistant locations.

Response to chemotherapy.

The *in vitro* cytotoxic effect of Cisplatin, Docetaxel and Paclitaxel (all obtained from Sigma-Aldrich; St. Louis, MO) were evaluated as previously described (26) in BG1 and HEY ovarian cancer cells. In brief, 2 X 10^4 cells that were transfected with control or *YY1*-specific siRNA oligos were treated with each drug for 24 hours beginning five hours post-transfection. The effect of treatment was measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay as described above. IC50 values (defined as 50% inhibition of cell proliferation) were calculated for each data point using GraphPad Prism 4 software (GraphPad Software, Inc.; San Diego, CA). Knockdown experiments, followed by chemosensitivity assays, were repeated ten times.

Statistical analysis.

For ranking of genes with or without TRANSFAC motifs, the Mann-Whitney U test was performed. Ranking of genes with or without the Gene Ontology cellular component annotation of microtubule was also analyzed using the Mann-Whitney U test and chi square tests where

appropriate. Survival comparison was performed using a Logrank test. Paired t-tests were used to compare IC50 values between control and *YY1* siRNA treated samples. Deviation of borderline tumors among clusters was calculated using Fisher's exact test. IC50 value differences between clusters in the NCI60 dataset were calculated using Mann-Whitney U tests. For these calculations, GraphPad Prism 4.0b software was used. The enrichment of Gene Ontology terms for ‘biological process’ was analyzed using GATHER (22). For other statistical analyses, two-tailed student’s t-tests were used. P values <0.05 were considered significant.

RESULTS

Comparison of *YY1* microarray expression with RT-PCR data, immunohistochemistry, and CGH arrays and potential epigenetic regulation.

Our prior microarray-based analysis of stage III-IV serous epithelial ovarian cancer specimens showed that *YY1* was one of the most differentially expressed genes that distinguished long-term (>7 years) from short-term (<3 years) survivors (1). We validated the accuracy of the microarray expression levels of *YY1* for 40 of these ovarian tumor specimens using quantitative real time RT-PCR (Figure S1A; r = -0.57, p=0.0001). We also examined YY1 expression by immunohistochemistry to verify expression in tumor cells and correlate this with mRNA expression. Expression of YY1 protein ranged from very low to very high, with localization of YY1 in the nucleus, as expected for this DNA binding protein. Higher nuclear YY1 expression in tumor cells was indeed associated with higher mRNA expression as detected by microarrays (r = 0.35, p=0.04, representative staining shown in Figure S1B).

To discern a cause for the differential expression of *YY1* in ovarian cancer with regard to survival, we investigated the possibility of genetic and/or epigenetic alterations at this locus in these cases. Chromosomal copy number changes are a frequent event in ovarian cancer and have been reported for the q arm of chromosome 14 where *YY1* is located (23-25). Such copy number changes might explain the differences in *YY1* expression observed between long and short term survivors. Comparative genomic hybridization (CGH) data was available for the majority of the ovarian tumors in our microarrayed dataset (unpublished data). We found that there is a significant relationship between the level of *YY1* expression detected by microarray analysis and the normalized signal intensities obtained for each of the genomic clones represented on the CGH arrays present at 14q32, evident for multiple 14q32 probes (Figure S1C). Promoter methylation and genomic imprinting were also analyzed for *YY1*, to explore

other possibile explanations for decreased expression in women with ovarian cancer living less than 3 years versus those living greater than seven years. Imprinting of *YY1* is plausible because of its location adjacent to a known imprinted cluser at 14q32 (Figure S2A). However, *YY1* was not imprinted (Figures S2B and S2C), nor was the *YY1* promoter CpG island subject to aberrant methylation in ovarian cancers (Figure S3).

Positive correlation between *YY1* expression and expression of genes containing YY1 and E2F binding motifs in ovarian tumors.

Gene expression correlations obtained from microarray data in isolation cannot define cause and effect relationships between gene transcripts. However, when gene expression data is coupled with transcription factor binding motif analysis, a relationship between effector and target can be inferred, especially when the effector regulates the transcription of other target genes. YY1 is known to have ubiquitous target sites, and to exert diverse effects on the transcription of downstream target genes. Our previously generated microarray dataset (1) was therefore analyzed for correlations in expression between *YY1* and genes positive for putative YY1 binding motifs (V$YY1_Q6, V$YY1_01 and V$YY1_02). Annotations for microarray probes were generated using the TRANSFAC database (19). We first ranked the expression values for the Affymetrix U133A probes (n=22,215) by their correlation to the expression value of the *YY1* probes and then created annotations for all Affymetrix probes that contain YY1 binding motifs. The results show that genes with YY1 binding motifs are significantly enriched among all genes showing positive correlation to *YY1* ($p<0.0001$ for each motif; results for V$YY1_Q6 shown in Figure 1A). This strongly suggests that YY1 primarily upregulates the expression of genes containing YY1 binding motifs in ovarian tumors.

Analysis of Gene Ontology (GO) terms (26) for the top 500 *YY1*-positively correlated genes with YY1 binding motif(s) using the web-based tool GATHER [Gene Annotation Tool to

Help Explain Relationships; (22)] showed enrichment of cell cycle-related GO terms (e.g. GO:0007049: cell cycle, GO:0007067: mitosis; $p<0.0001$, respectively). Furthermore, transcription factor E2F binding motifs were increased in this group of genes based on GATHER (data not shown). We therefore turned our attention to the relationship between YY1 and E2F. Among the U133A probes with an annotation for YY1 binding motifs (V$YY1_Q6, n=4,749), expression of genes with transcription factor E2F binding motifs (V$E2F_Q2, V$E2F1_Q3, V$E2F_Q6, V$E2F1_Q6, V$E2F_Q4, and V$E2F1_Q6_01) were also positively correlated to the expression of *YY1* in ovarian tumor tissue (motif positive versus motif negative, Mann-Whitney U test; $p<0.0001$ for each site; results for V$E2F1_Q3 shown in Figure 1B). The positive correlation of all three YY1 binding motifs with *YY1* expression, together with the presence of E2F sites among these genes suggests that YY1 upregulates expression of genes that contain both YY1 and E2F binding motifs.

Clustering of ovarian tumors by *YY1*-positively correlated, YY1 and E2F site-containing genes.

To better understand the relationship in ovarian cancer between tumor phenotype and *YY1*-correlated genes, we performed hierarchical clustering, using the top 250 *YY1*-positively correlated genes that contain YY1 and E2F binding motifs (V$YY1_Q6 and V$E2F1_Q3, respectively) and for which RMA-normalized expression values exceeded 7 in at least one tumor. We again found that cell cycle related GO terms were significantly enriched in this gene set (Table SI). Clustering of the ovarian tumors based on the expression of these 250 genes created two groups: the 'YY1 Low' cluster and the 'YY1 High' cluster (Figure 1C). The clusters are based on the low and high expression levels, respectively, of the constituent genes. Since E2F binding motifs characterize this gene set, we also examined the probability of the presence of the recently defined E2F3 gene signature (16) in each ovarian tumor. The predicted probability for having the E2F3 signature positively correlates with the expression of YY1

(r=0.59, p<0.0001, not shown) and was significantly different between the YY1 Low and YY1 High gene clusters (p<0.0001; Figure 1C, bottom). All ovarian borderline tumors were localized within the YY1 Low cluster (p<0.0001, Fisher's exact test) and also had very low probabilities of gene expression profiles constituting the E2F3 gene signature. These results show that the expression of putative YY1 target genes in ovarian cancer positively correlates with the probability of the tumor having an E2F3 gene signature.

Long-term survivors of advanced stage ovarian cancer clustered primarily with the tumors exhibiting higher expression of putative YY1 target genes, although this clustering was not statistically significant (median survival: 31 months versus 86 months). Different chemotherapeutic regimens were used among these patients, largely due to the addition of paclitaxel to the standard regimen only ten years ago (27, 28). We therefore restricted our analysis to patients who had received the current standard regimen consisting of paclitaxel and platinum to determine the influence of the YY1 signature on patient survival. Survival was only improved in the YY1 High cluster versus the YY1 Low cluster when primary ovarian cancer patients received chemotherapy that included paclitaxel (p=0.016; Figure 1D, left). In contrast, survival of primary ovarian cancer patients who did not receive paclitaxel was no different between the YY1 High and YY1 Low clusters. (p=0.47; Figure 1D, right).

To provide additional evidence that the 250 probes identified from our analysis represent putative downstream target genes for YY1, we analyzed an additional external microarray dataset consisting of 79 ovarian cancer specimens (2). In agreement with our initial results, genes with YY1 binding motifs were significantly enriched in positive correlation with *YY1* in the validation dataset (V$YY1_Q6; p<0.0001, V$YY1_01;p=0.005, V$YY1_02; p<0.0001). Among V$YY1_Q6(+) genes, E2F binding motifs were also enriched in positive correlation with *YY1* (for V$E2F_Q2, V$E2F1_Q3, V$E2F1_Q6, V$E2F_Q6, V$E2F1_Q6_01, and V$E2F_Q4;

p<0.0001 for all). Next, we analyzed the validation set using the same 250 probes generated from the initial analysis. These 250 probes also showed strong positive correlation with *YY1* in the external dataset (not shown; p<0.0001). Hierarchical clustering with respect to the 250 probes in the external dataset again generated two clusters with low and high expression of *YY1* and putative YY1 target genes (p<0.0001, Figure S4A). Survival analysis indicated that patients in the "YY1 High cluster" who were treated with paclitaxel showed improved survival as compared to the other groups (p=0.010, Figure S4B). Comparison of survival among "YY1 High" versus "YY1 Low" tumors from patients treated with paclitaxel shows improved survival of patients in the "YY1 High" group, but this did not quite reach statistical significance (p=0.14). Nevertheless, these findings demonstrate reproducibility between ovarian cancer datasets in detecting associations among YY1, its putative downstream targets, and survival of patients treated with or without paclitaxel.

Relationship between chemotherapeutic sensitivity and *YY1*-positively correlated genes among cancer cell lines in the NCI60 database.

To further validate our findings and determine if the association between *YY1* expression and taxane response was present in other types of cancer cells, we analyzed the NCI60 cell line data. The NCI60 database consists of a panel of 59 establlished cancer cell lines from multiple tissue types, with accompanying Affymetrix U95Av2 microarray data, for which pharmacologic response data has been generated for over 40,000 tested compounds (29). As we had found for ovarian cancer, when all probes (n=12,558) were sorted based on correlation to the expression level of *YY1*, V$YY1_Q6, V$YY1_01 and V$YY1_02 motif-positive genes were significantly enriched among genes with positive correlation to *YY1* (for V$YY1_Q6 and V$YY1_01, p<0.0001; for V$YY1_02, p=0.0004; data for V$YY1_Q6 shown in Figure 2A).

Further analysis of the NCI60 dataset revealed other similarities to the results obtained with the ovarian cancer microarray data. For example, among the U95Av2 probes with an annotation for YY1 binding motifs (V$YY1_Q6, n=2,537), expression of genes with E2F binding motifs (V$E2F_Q2, V$E2F1_Q3, V$E2F_Q6, V$E2F1_Q6, V$E2F_Q4, and V$E2F1_Q6_01) was found to also be positively correlated to the expression of *YY1* ($p<0.0001$ for each site; result for V$E2F1_Q3 shown in Figure 2B). Gene Ontology terms for this subgroup of genes (YY1- and E2F-site positive genes in positive correlation with YY1) also showed striking resemblance between the ovarian cancers and NCI60 cell lines, including cell cycle terms (Table SII).

Clustering was performed by taking the top 150 *YY1*-positively correlated probes with both YY1 and E2F binding motifs from the NCI60 dataset and dividing the genes into YY1 High and YY1 Low clusters. Supporting our results for the primary ovarian cancers, the NCI60 cancer cell lines in the YY1 High cluster were more sensitive to Paclitaxel and Docetaxel than cell lines in the YY1 Low cluster (Figure 2C). On the other hand, sensitivity to Cisplatin and Carboplatin, both platinum-based drugs, did not differ between the cell lines in these clusters.

Effect of *YY1* knockdown on ovarian cancer cell behavior.

To better understand the function of YY1 in ovarian cancer, we used siRNA-mediated knockdown to suppress *YY1* expression followed by assays to measure the effect on cell phenotype in ovarian cancer cell lines. Transcriptional suppression was confirmed to be ~90% or greater with two independent siRNA oligos as compared to *YY1* expression using a non-silencing siRNA oligo (Figure 3A, left panel), and this suppression was maintained at least through 96 hours post-transfection (not shown). That transcriptional repression was achieved with two independent siRNA oligos supports specificity of the effect to *YY1.* Western blotting

showed that YY1 protein expression was also repressed as a result of siRNA-mediated knockdown of *YY1* (Figure 3A, right panel).

YY1 knockdown led to a significant reduction in cell proliferation using both *YY1*-specific siRNA oligos in HEY ($p=0.03$ and $p=0.003$, respectively) and BG1 cells ($p<0.0005$ for both oligos; Figure 3B). *YY1* knockdown affected reduced expression of *CDC6*, an established YY1/E2F target gene (12), and *MCM5*, a putative YY1/E2F target gene (10, 30) (Figure 3C). *YY1* knockdown also resulted in suppression of anchorage-independent growth in BG1 and HEY cells ($p=0.002$ and $p=0.001$, respectively; Figure 3D) although these results may reflect the reduced proliferative capacity also induced by *YY1* suppression (refer to Figure 3B). Furthermore, *YY1* suppression reduced the motility of BG1 and HEY cells as measured by their ability to fill in a gap induced by scraping a monolayer of cells with a pipet tip (Figure 3E; at 28 hours, $p=0.0007$ and $p=0.0006$, respectively).

We then analyzed the effect of *YY1* knockdown on response to Paclitaxel, Docetaxel and Cisplatin by calculating the 50% Growth Inhibitory (IC50) dose for each drug in the HEY and BG1 ovarian cancer cell lines. The results for ten experiments each indicate that the IC50 was significantly increased for HEY and BG1 cells with *YY1* knockdown for both Paclitaxel ($p=0.0005$ and $p=0.002$, respectively) and Docetaxel ($p=0.0007$ and $p=0.0004$, respectively) (Figure 4A). However, *YY1* knockdown did not alter sensitivity to Cisplatin in either cell line ($p=0.52$ and $p=0.62$, respectively) (Figure 4B).

Transcriptional regulation of microtubule-related molecules by YY1/E2F3.

Given the increased sensitivity to microtubule stabilizing taxanes when YY1 expression is increased, we analyzed the relationship between microtubule-related molecules and YY1/E2F3 and the gene ontology annotations (cellular component) for “microtubule” (GO#5874)

and “microtubule associated complex” (GO#5875). Affymetrix probe annotations linking genes with these GO terms were obtained from the Affymetrix web site (http://www.affymetrix.com). Among the 17,136 genes represented by the Affymetrix U133v2 54,613 probes, 150 genes have the annotation GO#5874 “microtubule” and 3,932 genes have the annotation for V$YY1_Q6. Overlap of the annotations for “microtubule” and YY1 binding motifs was statistically significant; 35% (53/150) of “microtubule” positive genes have YY1 binding motifs, as compared to 23% (3,879/16,986) of “microtubule” negative genes (p=0.0002, Chi square test). 69% (104/150) of genes with E2F binding motifs (V$E2F_Q2) also significantly overlapped with “microtubule” positive genes versus 61% for “microtubule” negative genes (10,341/16,986; p=0.035). Similarly, genes with a “microtubule associated complex” GO annotation (n=56) were more likely to have YY1 and E2F binding motifs versus those without (V$YY1_Q6: 27% vs. 23%, V$E2F_Q2: 68% vs. 61%), although this was not statistically significant likely due to small sample size.

In order to examine if the expression of microtubule-related genes is associated with expression of YY1 and E2F3, we utilized recently published data in which the consequence of E2F3 overexpression in primary mammary epithelial cells was analyzed by gene expression microarray (16). Using Gene Set Enrichment Analysis (GSEA), we found that genes with GO annotations “microtubule” (GO#5874) and “microtubule associated complex” (GO#5875) were significantly enriched in cells overexpressing E2F3 (Figures S5A and B, FDR=0.160 and FDR=0.205, respectively). We then examined our primary ovarian cancer microarray data and found that the Affymetrix U133A gene probes with these same microtubule-related GO designations were significantly enriched among genes positively correlated to YY1 (Figure S5C, GO#5874 “microtubule” p=0.004; GO#5875 “microtubule associated complex” p=0.012, Mann-Whitney U test).

DISCUSSION

Gene expression correlations obtained from microarray data in isolation cannot define cause and effect relationships between gene transcripts. However, when gene expression data is combined with transcription factor binding motif analysis, a relationship between effector and target can be inferred, especially when the effector regulates the transcription of other target genes. YY1 is known to have ubiquitous target sites, and to exert diverse effects on the transcription of downstream target genes, depending on the nature of YY1 interactions with cofactors and its target sequence. We have shown here that *YY1* expression positively correlates with the expression of genes containing YY1-binding motifs in their promoter region in our ovarian cancer datasets, an external ovarian cancer dataset and in the NCI60 dataset. These results strongly suggest that YY1 upregulates these putative downstream target genes in cancer. However, this seems to differ with the known role of YY1 as both a transcriptional enhancer and suppressor of its downstream targets (9), since we did not find enrichment of YY1 binding motif-positive genes in negative correlation with *YY1*. This may be due to insufficient number of negatively regulated genes to detect by statistical analyses amid the background of the genome, or conversely, it may suggest that in cancer cells, the primary function of YY1 is to upregulate the expression of select target genes. These explanations are not mutually exclusive. Regardless, our results from analysis of the tumor microarrays and transcription factor binding motifs detected only *YY1*-correlated upregulation.

YY1 was previously shown to function synergistically with E2F2 and E2F3 to activate target gene transcription in a molecular complex bridged by the Ring1- and YY1-binding protein (RYBP) (12). Our results support that YY1 and E2F3 work together in regulating gene expression in ovarian cancer and among the NCI60 cancer cell lines. Experimentally, YY1 is known to deregulate the cell cycle in cancer cells (9). Using siRNA-mediated knockdown of *YY1*

in ovarian cancer cells, we found that suppression of YY1 leads to a reduction in cell proliferation. We therefore considered that YY1, along with coactivator E2F, have a stimulatory effect on the cell cycle in this setting. We thus designated genes having both YY1 and E2F binding-motifs that were positively correlated with *YY1* expression as putative YY1 target genes.

Clustering by the putative YY1 target genes clearly divided ovarian cancer into two distinct groups, and the expression of genes within these groups strikingly correlated with an independent gene expression profile that was generated by overexpressing E2F3 (23). This lends additional support to the idea that there is a strong association between YY1 and E2F activity in ovarian cancer. Further evidence of this association comes from our finding that borderline tumors, known to have low proliferative activity (31), are characterized by low expression of putative YY1 downstream target genes and low E2F3 activity.

In spite of the propensity of YY1/E2F to increase oncogenic behavior of cells *in vitro*, we found that a poorer prognosis among primary ovarian cancer patients was evident in the YY1 Low cluster rather than in the YY1 High cluster. In support of this, association between poor prognosis and low proliferative activity was previously shown in ovarian cancer (32-34). In the current study, the difference in prognosis for YY1 High versus YY1 Low clusters was statistically significant only in the group of patients treated with paclitaxel. Hence, prognoses may be determined by the ability of YY1 to modulate chemotherapeutic response, with high YY1 expression working to enhance the effectiveness of the drug through modulation of its downstream targets.

In order to further evaluate a potential role for YY1 in affecting chemotherapeutic response, we analyzed the NCI60 dataset. Correlations between *YY1* expression and genes with YY1 and E2F binding motifs in the NCI60 dataset were the same as those we observed in

ovarian cancer, and gene ontology terms of the putative YY1 target genes among the NCI60 cell lines also largely mimicked those in ovarian cancer. This indicates similarity of biological roles of YY1 in ovarian cancer and in the NCI60 cancer cell lines. Because of these shared features, we compared the survival of ovarian cancer patients with respect to chemotherapeutic treatment with the chemotherapeutic responses observed in the NCI60 cancer cell lines.

When the NCI60 cells were divided into two groups based on the expression of YY1/E2F putative target genes, the YY1 High cluster exhibited increased sensitivity to taxanes, but not to platinum based drugs. This result is similar to the ovarian cancer data in that the improved prognosis of ovarian cancer patients treated with paclitaxel is associated with the elevated expression of putative YY1/E2F downstream genes. These data strongly implicate YY1/E2F as an effector of response to taxane-based chemotherapeutic agents. This was confirmed with our finding that *YY1* knockdown reproducibly increases resistance to both paclitaxel and docetaxel, but not to cisplatin in ovarian cancer cell lines. This result is further supported by a previous report showing that overexpression of E2F increases sensitivity to paclitaxel but not to cisplatin (35).

The reasons underlying the association between taxane sensitivity and higher YY1/E2F activity remains unclear. As taxane specifically targets microtubules, the mechanism(s) of sensitivity could be related to microtubule function (36). Our finding that knockdown of YY1 suppressed both proliferation and migration negates our hypothesis that YY1 functions as a tumr suppressor, but supports the notion that YY1/E2F have a role in regulating microtubule function, since migration is known to have a strong association with microtubule dynamics (37). Several additional lines of evidence also suggest that YY1/E2F have a regulatory role in directing the transcription of proteins involved in microtubule dynamics, including 1) our computational analysis showing the increased expression of genes with YY1 and E2F binding

motifs that comprise the microtubule-related Gene Ontology data sets, 2) upregulation of microtubule-related genes by overexpression of E2F3, and 3) positive correlation between the expression of microtubule-related genes and *YY1* in ovarian cancer. It is not clear whether transcriptional upregulation of many microtubule-related genes by YY1/E2F caused taxane-specific sensitivity or not. However, our results may provide the foundation and impetus for further investigation of microtubule function and relationship to YY1 to determine the mechanistic basis of taxane sensitivity in YY1 High tumor cells.

In conclusion, we have used gene expression data from microarrays to elucidate molecular mechanisms involved in chemosensitivity. We found that YY1, in association with E2F, upregulates downstream genes in ovarian cancer. A “YY1 High” gene signature is associated with improved prognosis in ovarian cancer when patients are treated with taxanes. We also showed that high YY1 activity results in increased sensitivity to taxanes *in vitro*. These findings may be clinically applicable in distinguishing taxane-sensitive patients from taxane-resistant patients to more effectively individualize treatment of ovarian cancer and enhance survival.

REFERENCES

1. Berchuck A, Iversen ES, Lancaster JM*, et al.* Patterns of gene expression that characterize long-term survival in advanced stage serous ovarian cancers. Clin Cancer Res 2005;11(10):3686-96.

2. Spentzos D, Levine DA, Ramoni MF*, et al.* Gene expression signature with independent prognostic significance in epithelial ovarian cancer. J Clin Oncol 2004;22(23):4700-10.

3. Flanagan JR, Becker KG, Ennist DL*, et al.* Cloning of a negative transcription factor that binds to the upstream conserved region of Moloney murine leukemia virus. Mol Cell Biol 1992;12(1):38-44.

4. Hariharan N, Kelley DE, Perry RP. Delta, a transcription factor that binds to downstream elements in several polymerase II promoters, is a functionally versatile zinc finger protein. Proc Natl Acad Sci U S A 1991;88(21):9799-803.

5. Park K, Atchison ML. Isolation of a candidate repressor/activator, NF-E1 (YY-1, delta), that binds to the immunoglobulin kappa 3' enhancer and the immunoglobulin heavy-chain mu E1 site. Proc Natl Acad Sci U S A 1991;88(21):9804-8.

6. Shi Y, Seto E, Chang LS, Shenk T. Transcriptional repression by YY1, a human GLI-Kruppel-related protein, and relief of repression by adenovirus E1A protein. Cell 1991;67(2):377-88.

7. Pisaneschi G, Ceccotti S, Falchetti ML, Fiumicino S, Carnevali F, Beccari E. Characterization of FIII/YY1, a Xenopus laevis conserved zinc-finger protein binding to the first exon of L1 and L14 ribosomal protein genes. Biochem Biophys Res Commun 1994;205(2):1236-42.

8. Brown JL, Mucci D, Whiteley M, Dirksen ML, Kassis JA. The Drosophila Polycomb group gene pleiohomeotic encodes a DNA binding protein with homology to the transcription factor YY1. Mol Cell 1998;1(7):1057-64.

9. Gordon S, Akopyan G, Garban H, Bonavida B. Transcription factor YY1: structure, function, and therapeutic implications in cancer biology. Oncogene 2006;25(8):1125-42.

10. Affar el B, Gay F, Shi Y*, et al.* Essential dosage-dependent functions of the transcription factor yin yang 1 in late embryonic development and cell cycle progression. Mol Cell Biol 2006;26(9):3565-81.

11. Palko L, Bass HW, Beyrouthy MJ, Hurt MM. The Yin Yang-1 (YY1) protein undergoes a DNA-replication-associated switch in localization from the cytoplasm to the nucleus at the onset of S phase. J Cell Sci 2004;117(Pt 3):465-76.

12. Schlisio S, Halperin T, Vidal M, Nevins JR. Interaction of YY1 with E2Fs, mediated by RYBP, provides a mechanism for specificity of E2F function. Embo J 2002;21(21):5775-86.

13. Petkova V, Romanowski MJ, Sulijoadikusumo I*, et al.* Interaction between YY1 and the retinoblastoma protein. Regulation of cell cycle progression in differentiated cells. J Biol Chem 2001;276(11):7932-6.

14. Wang CC, Chen JJ, Yang PC. Multifunctional transcription factor YY1: a therapeutic target in human cancer? Expert Opin Ther Targets 2006;10(2):253-66.

15. Cam H, Dynlacht BD. Emerging roles for E2F: beyond the G1/S transition and DNA replication. Cancer Cell 2003;3(4):311-6.

16. Bild AH, Yao G, Chang JT*, et al.* Oncogenic pathway signatures in human cancers as a guide to targeted therapies. Nature 2006;439(7074):353-7.

17. Karolchik D, Baertsch R, Diekhans M*, et al.* The UCSC Genome Browser Database. Nucleic Acids Res 2003;31(1):51-4.

18. Pruitt KD, Tatusova T, Maglott DR. NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. Nucleic Acids Res 2005;33(Database issue):D501-4.

19. Matys V, Fricke E, Geffers R*, et al.* TRANSFAC: transcriptional regulation, from patterns to profiles. Nucleic Acids Res 2003;31(1):374-8.

20. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 1998;95(25):14863-8.

21. Subramanian A, Tamayo P, Mootha VK*, et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102(43):15545-50.

22. Chang J, Nevins J. GATHER: A Systems Approach to Interpreting Genomic Signatures. Bioinformatics 2006:Accepted for Publication.

23. Bandera CA, Takahashi H, Behbakht K*, et al.* Deletion mapping of two potential chromosome 14 tumor suppressor gene loci in ovarian carcinoma. Cancer Res 1997;57(3):513-5.

24. Cliby W, Ritland S, Hartmann L*, et al.* Human epithelial ovarian cancer allelotype. Cancer Res 1993;53(10 Suppl):2393-8.

25. Osborne RJ, Leech V. Polymerase chain reaction allelotyping of human ovarian cancer. Br J Cancer 1994;69(3):429-38.

26. Harris MA, Clark J, Ireland A*, et al.* The Gene Ontology (GO) database and informatics resource. Nucleic Acids Res 2004;32(Database issue):D258-61.

27. McGuire WP, Hoskins WJ, Brady MF*, et al.* Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. N Engl J Med 1996;334(1):1-6.

28. Parmar MK, Ledermann JA, Colombo N*, et al.* Paclitaxel plus platinum-based chemotherapy versus conventional platinum-based chemotherapy in women with relapsed ovarian cancer: the ICON4/AGO-OVAR-2.2 trial. Lancet 2003;361(9375):2099-106.

29. Scherf U, Ross DT, Waltham M*, et al.* A gene expression database for the molecular pharmacology of cancer. Nat Genet 2000;24(3):236-44.

30. Ohtani K, Iwanaga R, Nakamura M*, et al.* Cell growth-regulated expression of mammalian MCM5 and MCM6 genes mediated by the transcription factor E2F. Oncogene 1999;18(14):2299-309.

31. Halperin R, Zehavi S, Dar P*, et al.* Clinical and molecular comparison between borderline serous ovarian tumors and advanced serous papillary ovarian carcinomas. Eur J Gynaecol Oncol 2001;22(4):292-6.

32. Moreno CS, Matyunina L, Dickerson EB*, et al.* Evidence that p53-mediated cell-cycle-arrest inhibits chemotherapeutic treatment of ovarian carcinomas. PLoS ONE 2007;2(5):e441.

33. Itamochi H, Kigawa J, Sugiyama T, Kikuchi Y, Suzuki M, Terakawa N. Low proliferation activity may be associated with chemoresistance in clear cell carcinoma of the ovary. Obstet Gynecol 2002;100(2):281-7.

34. Hartmann LC, Sebo TJ, Kamel NA*, et al.* Proliferating cell nuclear antigen in epithelial ovarian cancer: relation to results at second-look laparotomy and survival. Gynecol Oncol 1992;47(2):191-5.

35. Russo AJ, Magro PG, Hu Z*, et al.* E2F-1 overexpression in U2OS cells increases cyclin B1 levels and cdc2 kinase activity and sensitizes cells to antimitotic agents. Cancer Res 2006;66(14):7253-60.

36. Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. Nat Rev Cancer 2004;4(4):253-65.

37. Honore S, Pasquier E, Braguer D. Understanding microtubule dynamics for improved cancer therapy. Cell Mol Life Sci 2005;62(24):3039-56.

FOOTNOTES

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Note: N. Matsumura and Z. Huang contributed equally to this work.

FIGURE LEGENDS

Figure 1. *YY1 target genes in ovarian cancer.*

(**A**) Genes whose expression is correlated with that of *YY1* are enriched in binding motifs for YY1. Color bar represents all Affymetrix U133A probes with a RefSeq annotation arranged by their correlation to the expression of *YY1* using the expression values from 88 ovarian tumors. Black and white bar represents the same distribution of probes, where a single black horizontal line indicates the presence of an YY1 binding motif (V$YY1_Q6) for that gene. (**B**) Among 4,749 genes that contain the V$YY1_Q6 binding motif, genes whose expression is correlated with that of *YY1* are enriched for E2F binding motifs (V$E2F1_Q3). (**C**) Clustering by putative YY1 target genes in ovarian tumors. The top 250 YY1-positively correlated genes were selected for clustering based on 1) the presence of both YY1 (V$YY1_Q6) and E2F (V$E2F1_Q3) binding motifs and 2) having at least one tumor with relatively high level expression of the gene (RMA normalized value >7). The 'YY1 Low' and 'YY1 High' clusters are characterized by low and high expression, respectively of *YY1* and *YY1*-correlated genes. Color bar below the heat map, probability of having an 'E2F3 signature'. (**D**) Kaplan-Meier analysis of primary ovarian cancer patients who received platinum and did (left; N=36) or did not (right; N=28) receive paclitaxel. Patients are stratified based on the assignment of their tumor to the 'YY1 Low' or 'YY1 High' cluster.

Figure 2. *YY1 target genes in the NCI60 cell lines.*

(**A**) Genes whose expression is correlated with that of *YY1* are enriched in binding sites for YY1. Color bar represents all U95Av2 probes with a RefSeq annotation arranged by their correlation to the expression of *YY1* using the expression values from the NCI60 cell lines. Black and white bar represents the same distribution of probes, where a single black horizontal line indicates the presence of an YY1 binding motif (V$YY1_Q6) for that gene. (**B**) Among 2,537 genes that

contain YY1 binding motifs, genes whose expression is correlated with that of *YY1* are enriched for E2F binding sites (V$E2F1_Q3). (**C**) Heatmaps showing expression of putative downstream target genes of YY1 (bottom) and relation to taxane sensitivity in the NCI60 dataset (top). The top 150 YY1-positively correlated genes (Affymetrix U95Av2) were analyzed that contain both YY1 (V$YY1_Q6) and E2F (V$E2F1_Q3) binding motifs and that have at least one cell line with a log2(MAS5) expression value ≥7. Columns, individual cell lines; rows, individual gene probes. Cell lines (left to right): SNB-75, U251, A498, SF-295, UACC-62, SN12C, UO-31, 786-0, SNB-19, OVCAR-3, OVCAR-4, SK-MEL-28, UACC-257, RXF-393, SF-268, DU-145, SKOV-3, IGROV1, MALME-3M, OVCAR-8, SK-MEL-5, HOP-92, CAKI-1, TK-10, PC-3, T-47D, OVCAR-5, HCT-116, KM12, SW-620, HCT-15, MCF7, HT29, NCI-H226, MOLT-4, CCRF-CEM, NCI/ADR-RES, MDA-MB-231/ATCC, HCC-2998, A549/ATCC, NCIH522, NCI-H23, LOX IMVI, NCI-H460, EKVX, NCI-H322M, SK-MEL-2, SR, BT-549, HS 578T, SF-539, HOP-62, M14, MDA-MB-435, COLO 205, HL-60(TB), ACHN, RPMI-8226 and K-562. The 'YY1 Low' and 'YY1 High' clusters are indicated above the heat map. The four colored rows above the heat map represent GI50 values for (top to bottom) paclitaxel, docetaxel, cisplatin and carboplatin. Blue, resistant; red, sensitive.

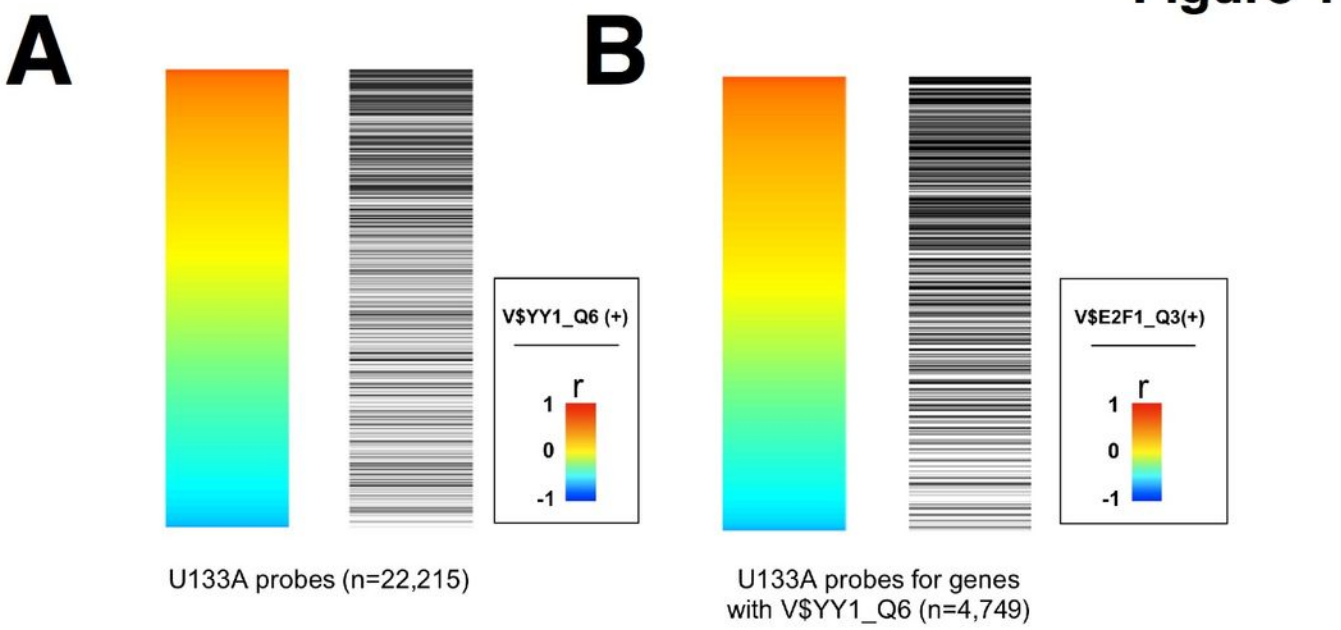
Figure 3. *Effect of YY1 knockdown on ovarian cancer cell behavior.*

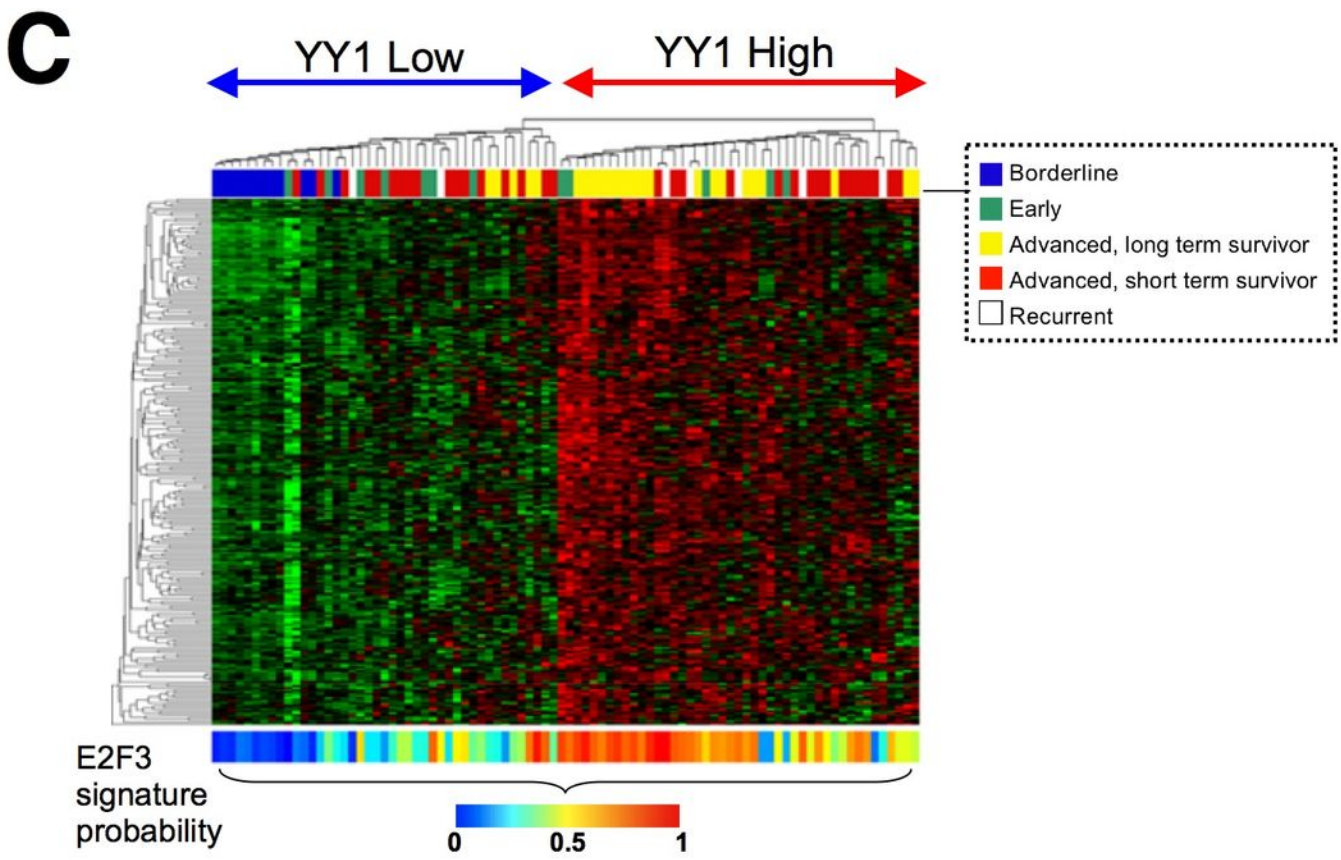
(**A**, left) Efficiency of siRNA-mediated knockdown of *YY1*. Cells were transfected with control non-silencing siRNA oligos (C) or with two independent siRNA oligos specific to *YY1* (1 and 2). Quantitative real-time RT-PCR assays were performed in duplicate and are shown for 72 hours post-transfection. Y-axis, expression (+SEM) relative to that of the control. **(A,** right) Western blotting of HEY and BG1 cells transfected with control or *YY1*-specific siRNAs at 48 and 72 hours post-transfection. GAPDH, endogenous control. (**B**) siRNA-mediated knockdown of *YY1* inhibits cell proliferation. Y-axis, mean absorbance (+SEM) at 490 nm at 72 hours post-transfection. (**C**) Suppression of YY1/E2F target genes *CDC6* and *MCM5* measured by

quantitative RT-PCR following knockdown of *YY1*. (**D**) Knockdown of *YY1* inhibits anchorage-independent growth. Results are shown (+SEM) for $2X10^3$ seeded cells per well; proportional results were obtained when $5X10^3$ cells were seeded per well. (**E**) Knockdown of *YY1* inhibits migration in wound healing assays. Photomicrographs were taken at the indicated time points following wounding.

Figure 4. *Knockdown of YY1 leads to increased resistance to paclitaxel and docetaxel (**A**), but not to cisplatin (**B**) in ovarian cancer cell lines.*

Figure 1


A
B
V$YY1_Q6 (+)
r
1
0
-1
V$E2F1_Q3(+)
r
1
0
-1
U133A probes (n=22,215)
U133A probes for genes
with V$YY1_Q6 (n=4,749)


C
YY1 Low
YY1 High
Borderline
Early
Advanced, long term survivor
Advanced, short term survivor
Recurrent
E2F3
signature
probability
0
0.5
1

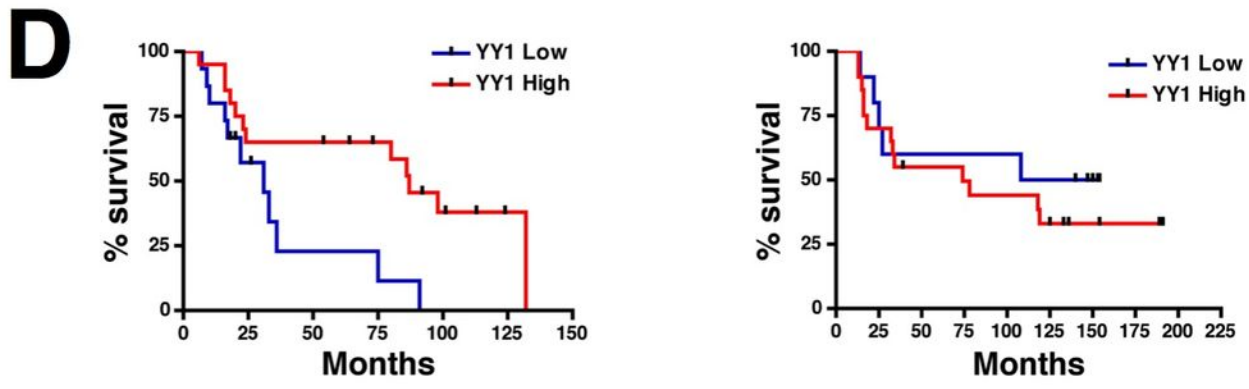
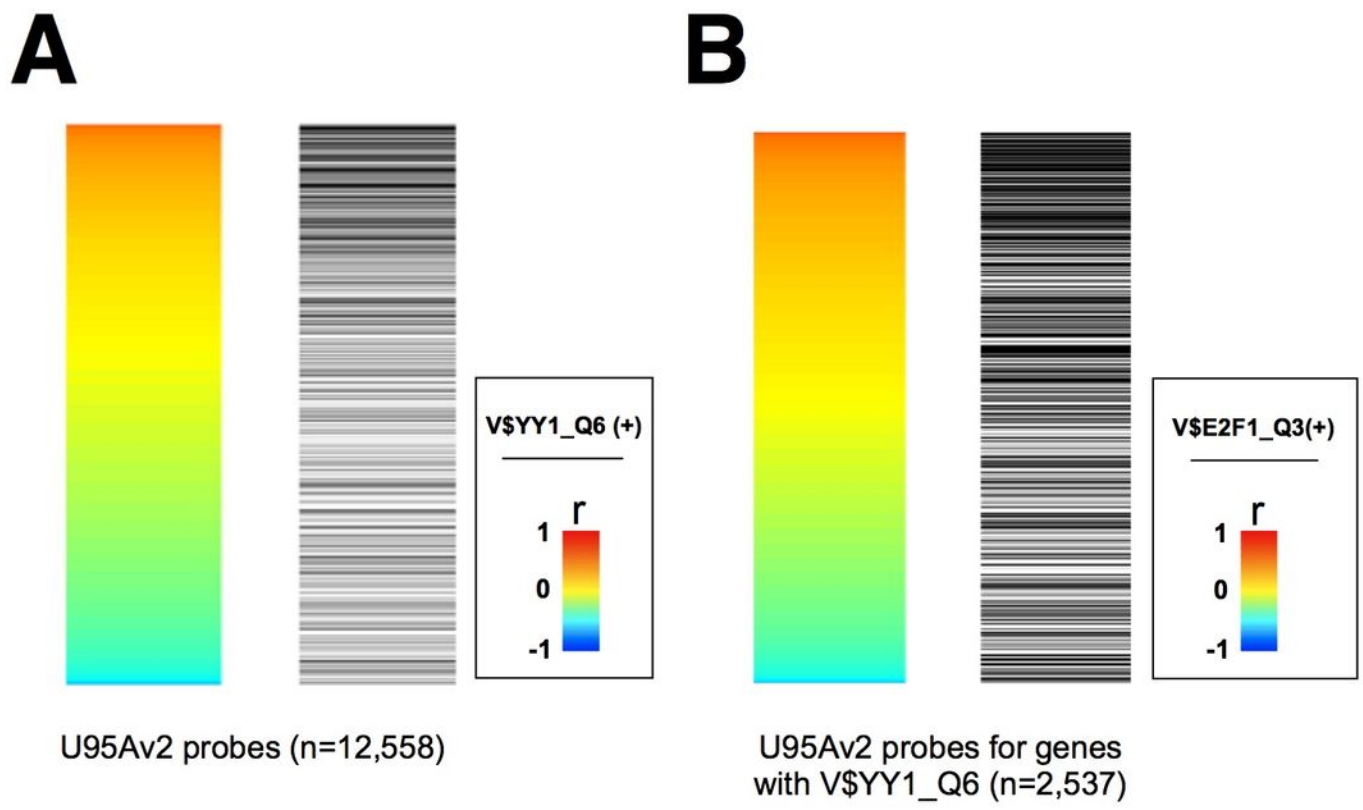

D
YY1 Low
YY1 High
% survival
Months
YY1 Low
YY1 High
% survival
Months

Figure 2


A
B
V$YY1_Q6 (+)
r
1
0
-1
V$E2F1_Q3(+)
r
1
0
-1
U95Av2 probes (n=12,558)
U95Av2 probes for genes
with V$YY1_Q6 (n=2,537)

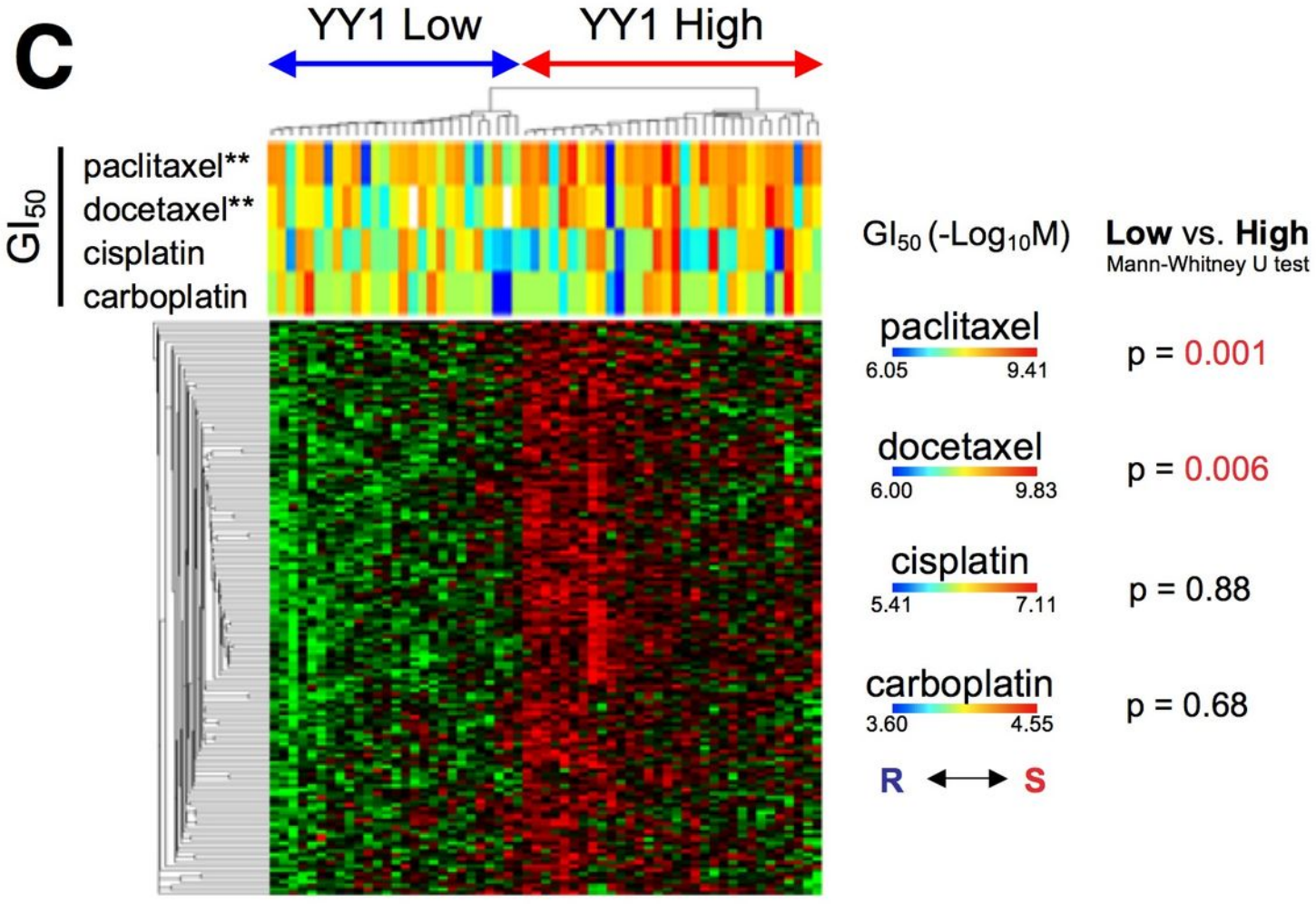

C
YY1 Low
YY1 High
GI50
paclitaxel**
docetaxel**
cisplatin
carboplatin
GI50 (-Log10M)
Low vs. High
Mann-Whitney U test
paclitaxel
6.05
9.41
p = 0.001
docetaxel
6.00
9.83
p = 0.006
cisplatin
5.41
7.11
p = 0.88
carboplatin
3.60
4.55
p = 0.68
R
S

Figure 3

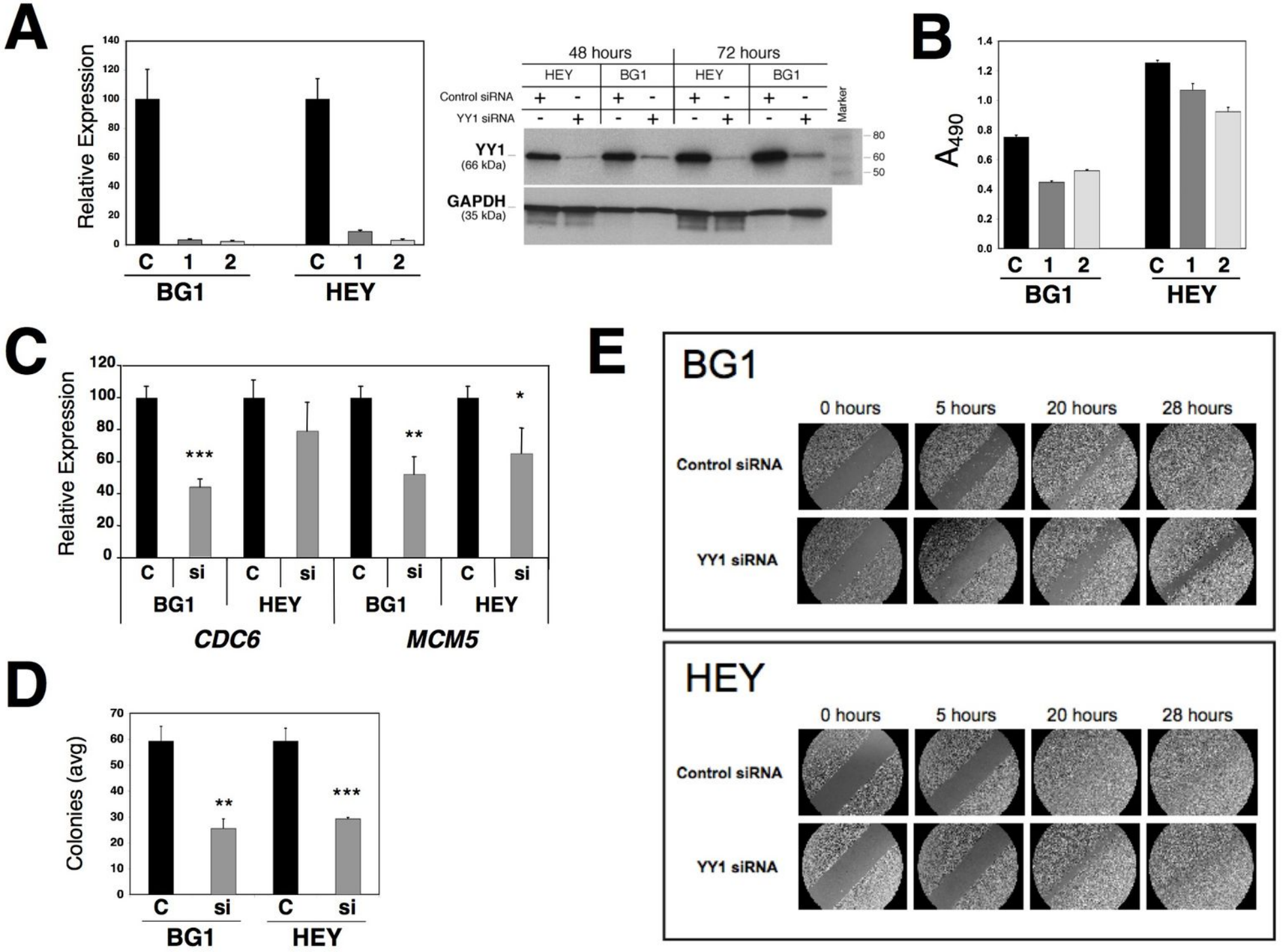
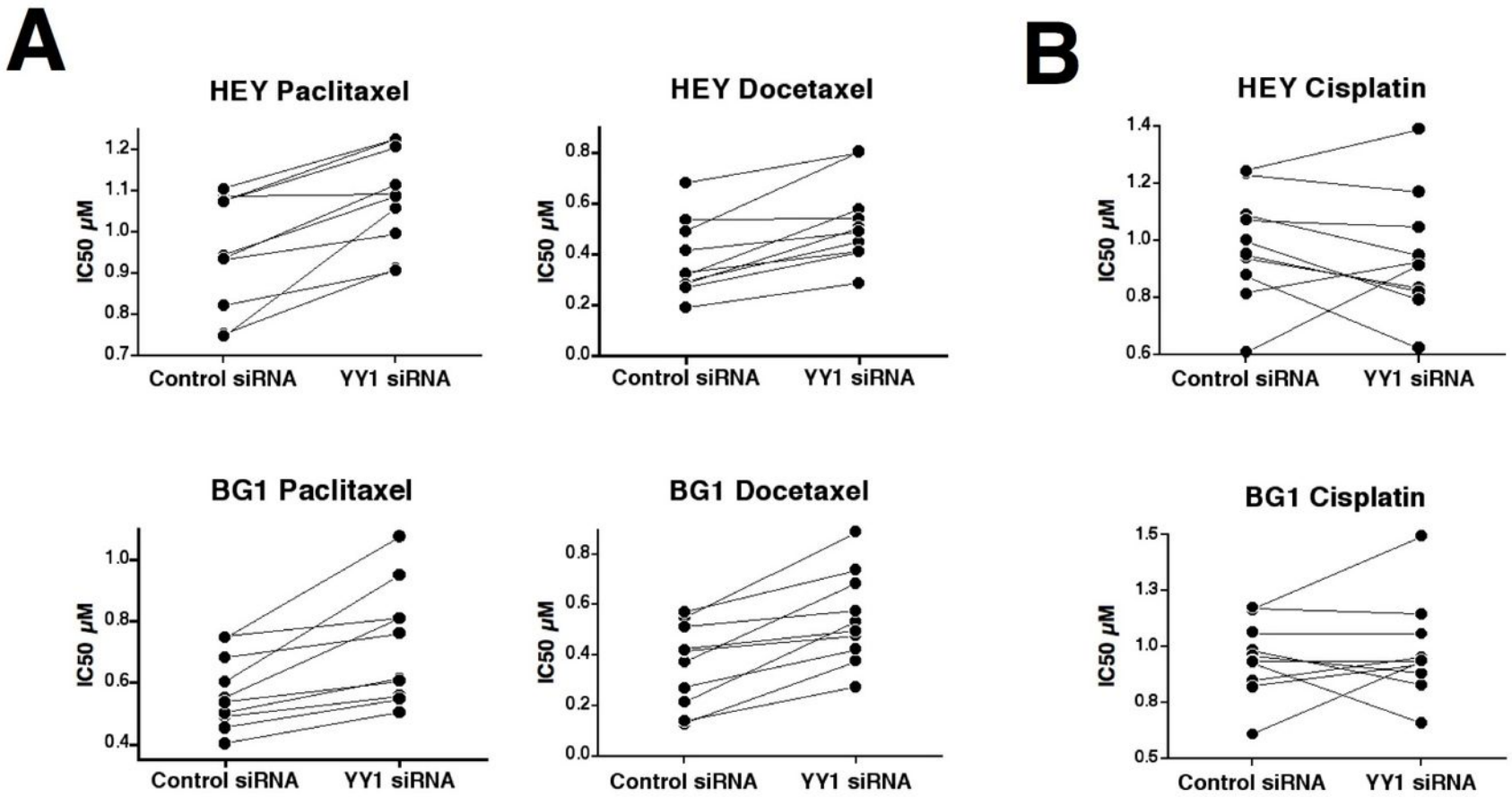

A
Relative Expression
140
120
100
80
60
40
20
0
C 1 2
BG1
C 1 2
HEY
48 hours
72 hours
HEY
BG1
HEY
BG1
Control siRNA + - + - + - + -
YY1 siRNA - + - + - + - +
Marker
YY1
(66 kDa)
GAPDH
(35 kDa)
80
60
50
B
A490
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C 1 2
BG1
C 1 2
HEY
C
Relative Expression
120
100
80
60
40
20
0
***
**
*
C si C si C si C si
BG1 HEY BG1 HEY
CDC6
MCM5
D
Colonies (avg)
70
60
50
40
30
20
10
0
**
***
C si C si
BG1 HEY
E
BG1
0 hours
5 hours
20 hours
28 hours
Control siRNA
YY1 siRNA
HEY
0 hours
5 hours
20 hours
28 hours
Control siRNA
YY1 siRNA

Figure 4


A
HEY Paclitaxel
IC50 μM
1.2
1.1
1.0
0.9
0.8
0.7
Control siRNA
YY1 siRNA
HEY Docetaxel
IC50 μM
0.8
0.6
0.4
0.2
0.0
Control siRNA
YY1 siRNA
BG1 Paclitaxel
IC50 μM
1.0
0.8
0.6
0.4
Control siRNA
YY1 siRNA
BG1 Docetaxel
IC50 μM
0.8
0.6
0.4
0.2
0.0
Control siRNA
YY1 siRNA
B
HEY Cisplatin
IC50 μM
1.4
1.2
1.0
0.8
0.6
Control siRNA
YY1 siRNA
BG1 Cisplatin
IC50 μM
1.5
1.3
1.0
0.8
0.5
Control siRNA
YY1 siRNA