

Yin Yang 1 Modulates Taxane Response in Epithelial Ovarian Cancer

Noriomi Matsumura,^{1,4} Zhiqing Huang,¹ Tsukasa Baba,^{1,4} Paula S. Lee,¹ Jason C. Barnett,¹ Seiichi Mori,³ Jeffrey T. Chang,³ Wen-Lin Kuo,⁵ Alison H. Gusberg,¹ Regina S. Whitaker,¹ Joe W. Gray,⁵ Shingo Fujii,⁴ Andrew Berchuck,^{1,3} and Susan K. Murphy^{1,2,3}

¹Division of Gynecologic Oncology/Department of Obstetrics and Gynecology and ²Department of Pathology, Duke University Medical Center; ³Duke Institute for Genome Sciences and Policy, Duke University, Durham, North Carolina; ⁴Department of Gynecology and Obstetrics, Kyoto University, Kyoto, Japan; and ⁵Lawrence Berkeley National Laboratory, Berkeley, California

Abstract

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. Epigenetic and genetic characteristics of *YY1* in serous epithelial ovarian cancer were analyzed along with *YY1* mRNA and protein. Patterns of gene expression in primary serous epithelial ovarian cancer and in the NCI60 database were investigated using computational methods. *YY1* function and modulation of chemotherapeutic response *in vitro* was studied using small interfering RNA knockdown. Microarray analysis showed strong positive correlation between expression of *YY1* and genes with *YY1* and transcription factor E2F binding motifs in ovarian cancer 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 cross-linking 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. 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. (Mol Cancer Res 2009;7(2):210–20)

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.⁶ 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 cross-links. The majority of women with advanced-stage ovarian cancer succumb within 5 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 data set (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 homologues present in *Drosophila* (8). *YY1* protein has divergent cellular functions, including the ability to activate and repress gene

Received 6/2/08; revised 9/23/08; accepted 10/17/08; published OnlineFirst 02/10/2009.

Grant support: University of Alabama Specialized Program of Research Excellence in Ovarian Cancer Research grant P50 CA 083591 and Gail Parkins Ovarian Awareness Foundation (S.K. Murphy); National Cancer Institute grant T32 CA 093245 (P.S. Lee); Director of Office of Science, Office of Basic Energy Sciences of the U.S. Department of Energy contract DE-AC02-05CH11231, U.S. Army Medical Research and Materiel Command grant BC 061995, NIH, National Cancer Institute grants P50 CA 58207, P50 CA 83639, P30 CA 82103, U54 CA 112970, U24 CA 126477, and P01 CA 64602, National Human Genome Research Institute grant U24 CA 126551, and SmithKline Beecham (J.W. Gray). N. Matsumura and Z. Huang contributed equally to this work.

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Requests for reprints: Susan K. Murphy, Duke University Medical Center, 2185 F-CIEMAS, 101 Science Drive, Box 91012, Durham, NC 27708. Phone: 919-681-3423; Fax: 919-684-5336. E-mail: murph035@mc.duke.edu

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doi:10.1158/1541-7786.MCR-08-0255

⁶ <http://www.cancer.gov>

transcription, modulate the function of other proteins, and affect chromatin structure (9). *YY1* transcription and function are also tied to progression of the cell cycle (10-13), implicating a possible role in carcinogenesis (9, 14). *YY1* was shown previously to function synergistically with E2F2 and E2F3 to activate target gene transcription in a molecular complex bridged by the Ring1- and YY1-binding protein (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 data set comprising 59 cell lines of multiple cancer types, we show herein that *YY1* in association with E2F up-regulates 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.

Results

Comparison of YY1 Microarray Expression with Reverse Transcription-PCR Data, Immunohistochemistry, and Comparative Genomic Hybridization Arrays and Potential Epigenetic Regulation

Our prior microarray-based analysis of stage III to 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 reverse transcription-PCR (Supplementary Fig. 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 Supplementary Fig. 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 (16-18). Such copy number changes might explain the differences in *YY1* expression observed between long-term and short-term survivors. Comparative genomic hybridization data were available for the majority of the ovarian tumors in our microarrayed data set.⁷ 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 comparative genomic hybridization arrays present at 14q32, evident for multiple 14q32 probes (Supplementary Fig. S1C). Promoter methylation and genomic imprinting were also analyzed for *YY1* to explore other possible explanations for decreased expression in women with ovarian cancer living <3 years versus those living >7 years. Imprinting of *YY1* is plausible because of its location adjacent to a known imprinted cluster at 14q32 (Supplementary Fig. S2A). However, *YY1* is not imprinted (Supplementary Fig. S2B and C), nor is the *YY1* promoter CpG island subject to aberrant methylation in ovarian cancers (Supplementary Fig. 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 are 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 genes. *YY1* is known to have ubiquitous target sites and to exert diverse effects on the transcription of downstream genes. Our previously generated gene expression microarray data (1) were 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 Fig. 1A). This strongly suggests that *YY1* primarily up-regulates the expression of genes containing *YY1* binding motifs in ovarian tumors.

Analysis of Gene Ontology (GO) terms (20) for the top 500 *YY1* positively correlated genes with *YY1* binding motif(s) using the web-based Gene Annotation Tool to Help Explain Relationships (21) showed enrichment of cell cycle-related GO terms (e.g., GO:0007049: cell cycle and GO:0007067: mitosis; $P < 0.0001$ for both). Furthermore, transcription factor E2F binding motifs were increased in this group of genes based on this analysis (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) was 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 Fig. 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*

⁷ W.-L. Kuo, J. Frilyand, K. Smith-McCune, P. Shaw, P. Glenn, C. Zaloudek, M. Deavers, A. Berchuck, A. Jain, D. Gershenson, D. Huntsman, P. Spellman, G. Mills and J.W. Gray, unpublished data.

up-regulates 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 (Supplementary 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 (Fig. 1C). The

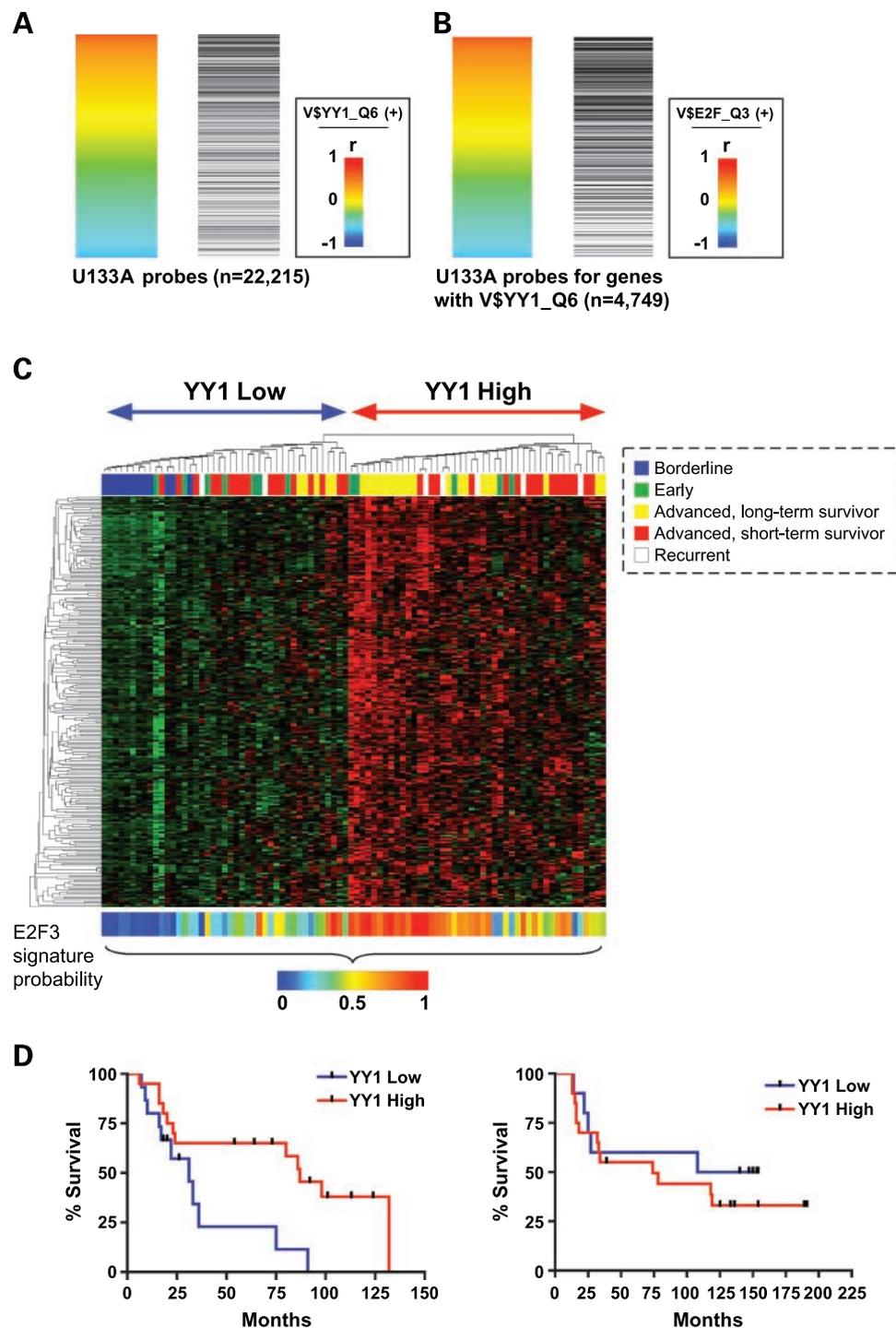


FIGURE 1. YY1 target genes in ovarian cancer. **A.** Genes whose expression is correlated with that of YY1 are enriched in binding motifs for YY1. Color bar, all Affymetrix U133A probes with a RefSeq annotation arranged by their correlation to the expression of YY1 using the expression values from 88 ovarian tumors. Black and white columns, same distribution of probes, where a single black horizontal line indicates the presence of a 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 (a) the presence of both YY1 (V\$YY1_Q6) and E2F (V\$E2F1_Q3) binding motifs and (b) 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.

clusters are based on the low and high expression levels, respectively, of the constituent genes. Because E2F binding motifs characterize this gene set, we also examined the probability of the presence of the recently defined E2F3 gene signature (22) in each ovarian tumor. The predicted probability for having the E2F3 signature positively correlates with the expression of YY1 ($r = 0.59$, $P < 0.0001$; data not shown) and was significantly different between the YY1 Low and YY1 High gene clusters ($P < 0.0001$; Fig. 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 versus 86 months). Different chemotherapeutic regimens were used among these patients largely due to the addition of paclitaxel to the standard regimen only 10 years ago (23, 24). We therefore analyzed patients who had received the current standard regimen consisting of paclitaxel and platinum versus those that did not receive paclitaxel 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 ($P = 0.016$; Fig. 1D, *left*). In contrast, survival of primary ovarian cancer patients who did not receive paclitaxel was no different between the YY1 High and the YY1 Low clusters ($P = 0.47$; Fig. 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 data set consisting of 79 ovarian cancer specimens (2). In agreement with our initial results, genes with YY1 binding motifs were significantly enriched among those in positive correlation with YY1 in the validation data set (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 genes with positive correlation to 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 data set (data not shown; $P < 0.0001$). Hierarchical clustering with respect to the 250 probes in the external data set again generated two clusters with low and high expression of YY1 and putative YY1 target genes ($P < 0.0001$; Supplementary Fig. S4A). Survival analysis indicated that patients in the YY1 High cluster who were treated with paclitaxel showed improved survival compared with the other groups ($P = 0.010$; Supplementary Fig. 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 show reproducibility between ovarian cancer data sets in detecting associations among YY1, its putative downstream targets, and survival of patients treated with 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 established cancer cell lines from multiple tissue types, with accompanying Affymetrix U95Av2 microarray data, for which pharmacologic response data have been generated for >40,000 tested compounds (25). As we have 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 Fig. 2A).

Further analysis of the NCI60 data set 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 Fig. 2B). GO 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 (Supplementary Table SII).

Clustering was done by taking the top 150 YY1 positively correlated probes with both YY1 and E2F binding motifs from the NCI60 data set 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 (Fig. 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 small interfering RNA (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 $\geq 90\%$ with two independent siRNA oligos compared with YY1 expression using a nonsilencing siRNA oligo (Fig. 3A, *left*), and this suppression was maintained at least through 96 h post-transfection (data not shown). That transcriptional repression was achieved with two independent siRNA oligos supports specificity of the effect to YY1. Western blotting showed that YY1 proteins expression was also repressed as a result of siRNA-mediated knockdown of YY1 (Fig. 3A, *right*).

YY1 knockdown led to a significant reduction in cell proliferation using both *YY1*-specific siRNA oligos in HEY ($P = 0.03$ and 0.003 , respectively) and BG1 ($P < 0.0005$ for both oligos; Fig. 3B) cells. *YY1* knockdown affected reduced expression of *CDC6*, an established *YY1*/E2F target gene (12), and *MCM5*, a putative *YY1*/E2F target gene (refs. 10, 26; Fig. 3C). *YY1* knockdown also resulted in suppression of anchorage-independent growth in BG1 and HEY cells ($P = 0.002$ and 0.001 ,

respectively; Fig. 3D), although these results may reflect the reduced proliferative capacity also induced by *YY1* suppression (refer to Fig. 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 pipette tip (Fig. 3E; at 28 h, $P = 0.0007$ and 0.0006 , respectively).

We then analyzed the effect of *YY1* knockdown on response to paclitaxel, docetaxel, and cisplatin by calculating the IC_{50} for

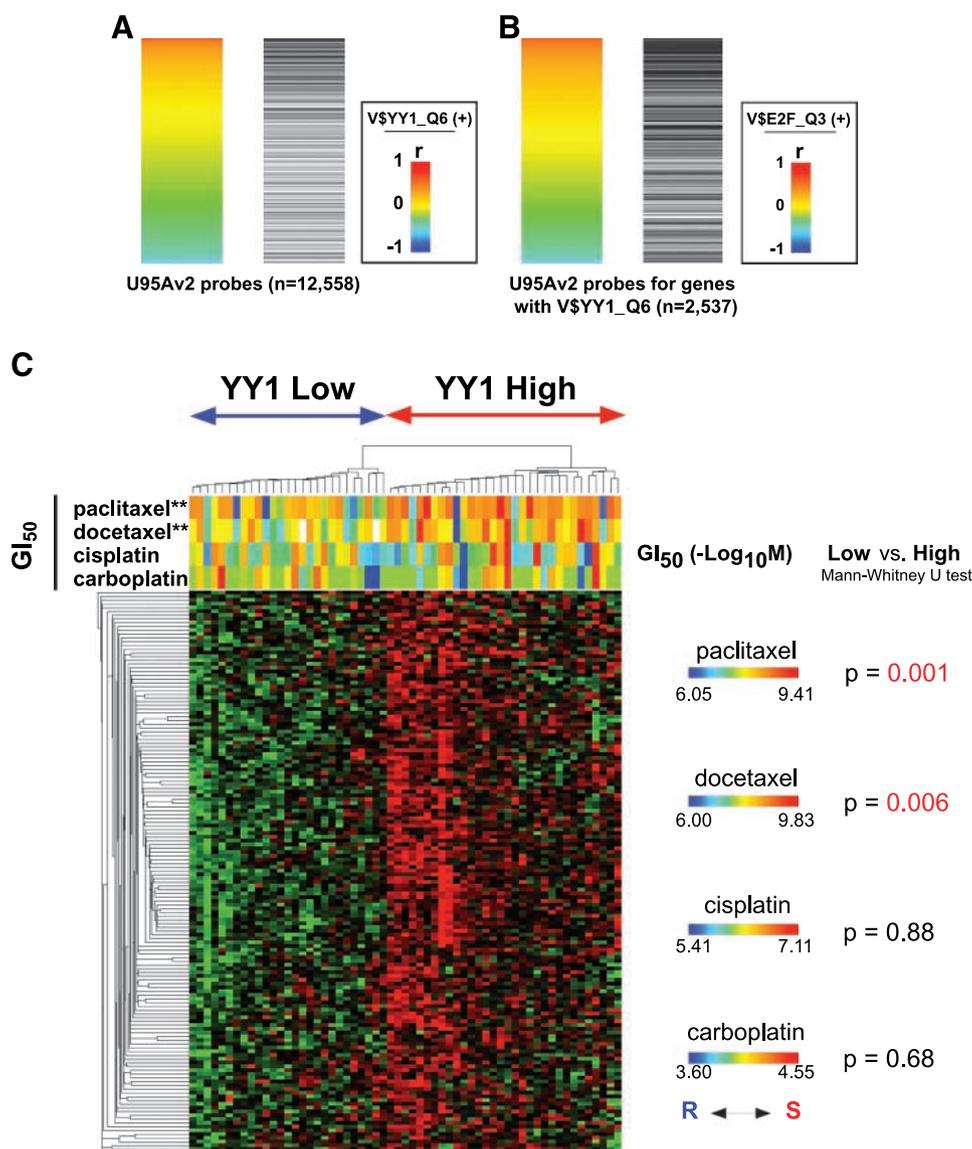


FIGURE 2. *YY1* target genes in the NCI60 cell lines. **A.** Genes whose expression is correlated with that of *YY1* are enriched in binding sites for *YY1*. Color bar, all U95Av2 probes with a RefSeq annotation arranged by their correlation to the expression of *YY1* using the expression values from the NCI60 cell lines. Black and white columns, same distribution of probes, where a single black horizontal line indicates the presence of a *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 data set (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 \log_2 (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, NCI-H522, NCI-H23, LOX IMVI, NCI-H460, EK VX, 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. Four colored rows above the heat map, GI_{50} values for (top to bottom) paclitaxel, docetaxel, cisplatin, and carboplatin. Blue, resistant; red, sensitive.

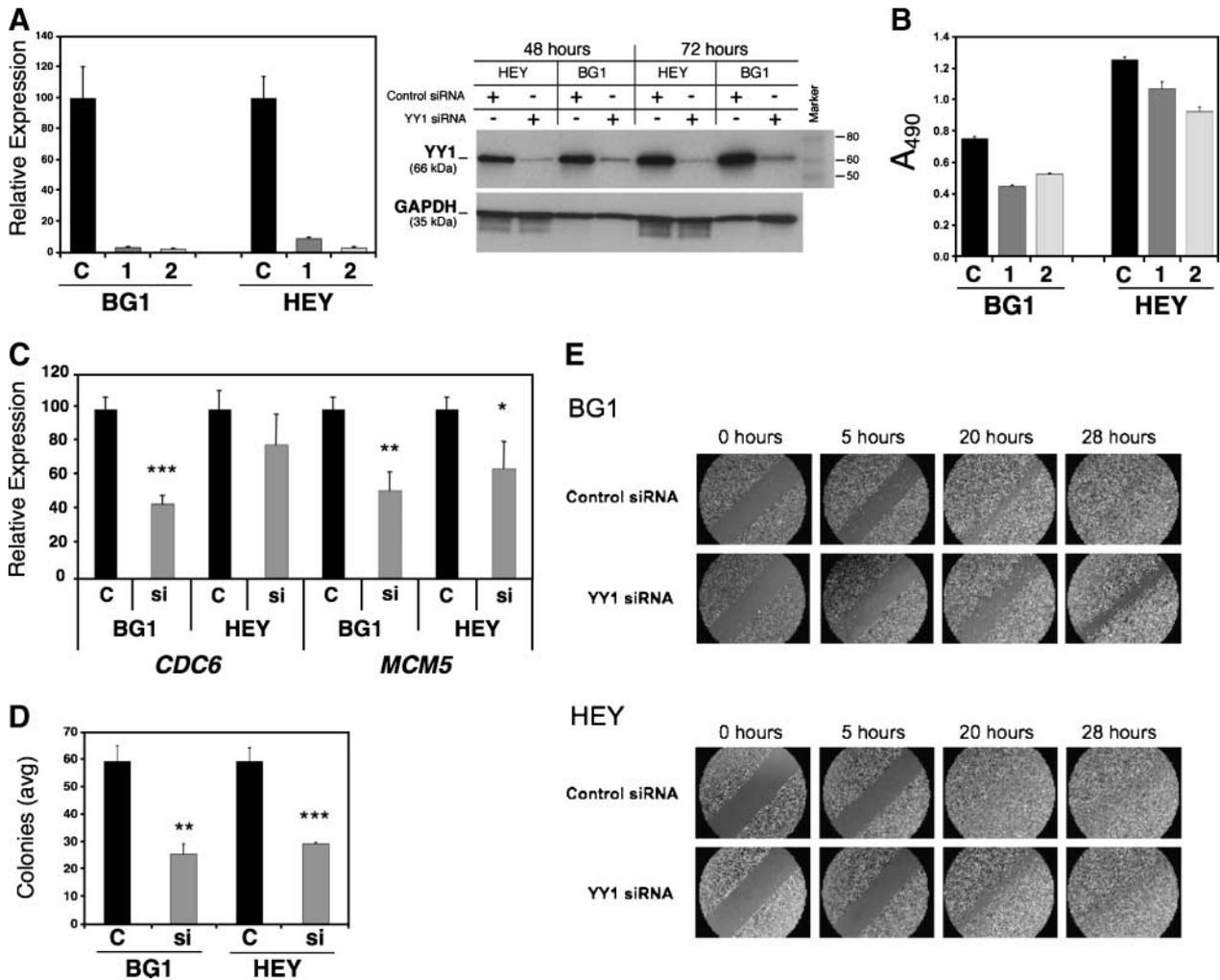


FIGURE 3. Effect of YY1 knockdown on ovarian cancer cell behavior. **A.** Left, efficiency of siRNA-mediated knockdown of YY1. Cells were transfected with control nonsilencing siRNA oligos (C) or with two independent siRNA oligos specific to YY1 (1 and 2). Quantitative real-time reverse transcription-PCR assays were done in duplicate and are shown for 72 h post-transfection. *Y axis*, expression \pm SE relative to that of the control. Right, Western blotting of proteins from HEY and BG1 cells transfected with control or YY1-specific siRNAs at 48 and 72 h post-transfection. GAPDH, endogenous control. **B.** siRNA-mediated knockdown of YY1 inhibits cell proliferation. *Y axis*, mean \pm SE absorbance at 490 nm at 72 h post-transfection. **C.** Suppression of YY1/E2F target genes *CDC6* and *MCM5* measured by quantitative reverse transcription-PCR following knockdown of YY1. **D.** Knockdown of YY1 inhibits anchorage-independent growth. Results \pm SE for 2×10^3 seeded cells per well; proportional results were obtained when 5×10^3 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.

each drug in the HEY and BG1 ovarian cancer cell lines. The results for 10 experiments each indicate that the IC_{50} was significantly increased for HEY and BG1 cells with YY1 knockdown for both paclitaxel ($P = 0.0005$ and 0.002 , respectively) and docetaxel ($P = 0.0007$ and 0.0004 , respectively; Fig. 4A). However, YY1 knockdown did not alter sensitivity to cisplatin in either cell line ($P = 0.52$ and 0.62 , respectively; Fig. 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 GO annotations (cellular component) for “microtubule” (GO5874) and “microtubule-associated complex” (GO5875). Affymetrix probe annotations linking genes with these GO terms were obtained from the Affymetrix Web site.⁸ Among the 17,136 genes represented by the 54,613 Affymetrix U133v2 probes, 150 genes have the annotation GO5874 “microtubule” and 3,932 genes have the annotation for VSYY1_Q6. Overlap of the annotations for “microtubule” and YY1 binding motifs was statistically significant; 35% (53 of 150) of “microtubule”-positive genes have YY1 binding motifs compared with 23% (3,879 of 16,986)

⁸ <http://www.affymetrix.com>

of “microtubule”-negative genes ($P = 0.0002$, χ^2 test). Sixty-nine percent (104 of 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 of 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% versus 23% and V\$E2F_Q2: 68% versus 61%), although this was not statistically significant likely due to small sample size.

To examine if the expression of microtubule-related genes is associated with expression of YY1 and E2F3, we used recently published data in which the consequence of E2F3 overexpression in primary mammary epithelial cells was analyzed by gene expression microarray (22). Using Gene Set Enrichment Analysis, we found that genes with GO annotations “microtubule” (GO5874) and “microtubule-associated complex” (GO5875) were significantly enriched in cells overexpressing E2F3 (Supplementary Fig. S5A and B; false discovery rate = 0.160 and 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 (Supplementary Fig. S5C; GO5874 “microtubule” $P = 0.004$; GO5875 “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 are 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 data set, an external ovarian cancer data set and the NCI60 data set. These results strongly suggest that YY1 up-regulates these putative downstream target genes in cancer. However, this seems to differ with the known role of YY1 as both a transcriptional enhancer and a suppressor of its downstream targets (9), because 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. Conversely, it may suggest that, in cancer cells, the primary function of YY1 is to up-regulate 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 up-regulation.

YY1 was shown previously to function synergistically with E2F2 and E2F3 to activate target gene transcription in a molecular complex bridged by the Ring1- and YY1-binding protein (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

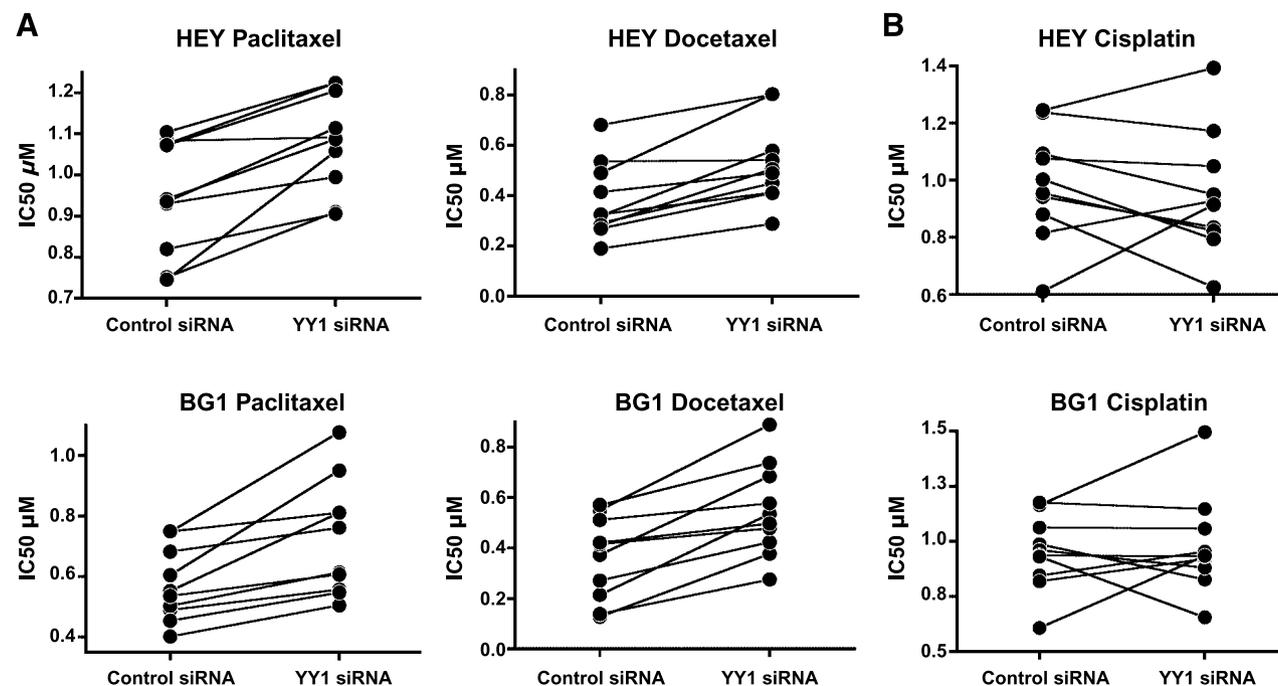


FIGURE 4. Knockdown of YY1 leads to increased resistance to paclitaxel and docetaxel (A) but not to cisplatin (B) in ovarian cancer cell lines.

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, has 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 (22). 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 (27), 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 shown previously in ovarian cancer (28-30). 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 activation of its downstream targets.

To further evaluate a potential role for YY1 in affecting chemotherapeutic response, we analyzed the NCI60 data set. Correlations between *YY1* expression and genes with YY1 and E2F binding motifs in the NCI60 data set were the same as those we observed in ovarian cancer, and GO 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 (31).

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 (32). Our finding that knockdown of YY1 suppressed both proliferation and migration negates our hypothesis that YY1 functions as a tumor suppressor but supports the notion that YY1/E2F have a role in regulating microtubule function, because migration is known to have a strong association with microtubule dynamics (33). 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 (a) our computational analysis showing the increased expression of genes with YY1 and E2F binding motifs that are members of the microtubule-related GO data sets, (b) up-regulation of microtubule-related genes by overexpression of E2F3, and (c) positive correlation between the expression of microtubule-related genes and *YY1* in ovarian cancer. It is not clear whether transcriptional up-regulation of many microtubule-related genes by YY1/E2F caused taxane-specific sensitivity. 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, up-regulates 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.

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 conceptual tissues were provided by the NIH-supported Laboratory of Human Embryology at the University of Washington at 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 (Invitrogen) with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin solution (Invitrogen) in a 37°C humidified incubator with 5% atmospheric CO₂.

Microarray Data Sets

Microarray data (Affymetrix U133A genechips) were generated and normalized as described previously (1). These microarray data include 12 serous borderline tumors, 12 early-stage (stage I/II) serous epithelial ovarian cancers, 24 advanced-stage (III/IV) serous ovarian cancers (living ≥7 years post-diagnosis), 33 advanced-stage serous ovarian cancers (living ≤3 years post-diagnosis), and 7 advanced-stage recurrent serous epithelial ovarian cancers. The clinicopathologic features of this patient population were described previously (1). We used microarray gene expression data from

an additional 79 advanced-stage serous epithelial ovarian cancers for external validation. This data set is derived from previously published data (22) by excluding samples overlapping with the above data set (1). NCI60 data from Affymetrix U95Av2 microarrays was also used.⁹

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 University of California-Santa Cruz Genome Browser (34, 35). 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 data set analysis following REDuction of Invariant probes (kindly performed by Expression Analysis; Supplementary Table S1). Reduction of Invariant probes 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 data set and the external validation data set. Pearson product-moment correlation coefficients to *YY1* were calculated for all the probes using the correlation (CORREL) function in EXCEL for each data set.

Clustering and Heat Maps

Clustering was done using Cluster 3.0.¹⁰ Genes for clustering were selected as described above. Before clustering, gene expression was median-centered and normalized in Cluster 3.0. Heat maps for expression microarray data were made with Java TreeView.¹¹ Heat maps for correlation coefficients and E2F3 signature probability data were made using the statistical computing and graphics environment, R.¹² 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 (36). Of 22,215 probes on the Affymetrix U133A genechip, ~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, respec-

tively) were selected for clustering after median-centered normalization using Cluster. For NCI60 U95Av2 data set, the top 150 probes were selected and clustering was conducted in the same way.

E2F3 Signature Probability

The E2F3 overexpression gene signature was developed previously using microarray data from recombinant adenovirus-infected primary mammary epithelial cells (22). This microarray data set was also used to analyze transcriptional regulation of microtubule related genes by E2F3.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis was done using Gene Set Enrichment Analysis software (37).¹³ The Affymetrix U133 probe lists having GO designations of “microtubule” (GO5874) or “microtubule-associated complex” (GO5875) were generated from the annotation files available from the Affymetrix Web site.⁸ Enrichment of these gene lists was examined between 10 control and 9 E2F3-overexpressing samples (22). 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 1×10^5 per well followed by transfection with 5 nmol/L of either control nonsilencing siRNA oligos or *YY1*-specific siRNA oligos (Qiagen) using HiPerfect reagent (Qiagen) according to the manufacturer's recommendations. *YY1*-specific siRNA oligo sequences were as follows: siRNA1 target sequence GAC-GACGACTACATTGAACAA, siRNA1 sense oligo r(CGAC-GACUACAUUGAACAA)dTdT, siRNA1 antisense oligo r(UUGUUCAAUGUAGUCGUCG)dTdT, siRNA2 target sequence AACCTGAAATCTCACATCTTA, siRNA2 sense oligo r(CCUGAAAUCUCACAUCUUA)dTdT, and siRNA2 antisense oligo r(TAAGATGTGAGATTTTCAGG)dTdT. The cells were trypsinized 24 h 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 h 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, and 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 $\beta 2$ -microglobulin, intended as a control for input RNA normalization (TaqMan Assays-on-Demand, Assay ID Hs00187842_m1). All reverse transcription reactions were done with 1 μ g total RNA as input measured on a Nanodrop 1000 spectrophotometer (Thermo Scientific). We therefore

⁹ <http://www.dtp.nci.nih.gov/>

¹⁰ <http://rana.lbl.gov/EisenSoftware.htm>

¹¹ <http://jtreeview.sourceforge.net/>

¹² <http://www.rproject.org/>

¹³ <http://www.broad.mit.edu/gsea/>

directly used cycle threshold values for all assessments of YY1 mRNA levels in real-time reverse transcription-PCR experiments, which were done 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 h before harvesting. Whole-cell lysates were prepared using Biosource NP-40 cell lysis buffer (Invitrogen). Proteins were resolved by SDS-PAGE (4-15%) and transferred to a nitrocellulose membrane (Bio-Rad). A 1:200 dilution of mouse anti-human YY1 antibody (H-10; Santa Cruz Biotechnology) was used followed by detection with a 1:3,000 dilution of secondary antibody (Bio-Rad) and the Enhanced Chemiluminescence system (Amersham Biosciences). Mouse anti-GAPDH antibody was used as an internal loading control.

Cell Proliferation Assays

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

Anchorage-Independent Growth Assays

BG1 or HEY ovarian cancer cells (10^3) 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 to 10 days before counting colonies >100 μ m 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. Twenty-four 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 h after transfection. "Wounds" were introduced through the cell monolayer at 72 h post-transfection using a P-1000 pipette tip. The cells were gently rinsed with PBS and cultured in RPMI 1640 containing 10% fetal bovine serum. Micrographs were taken at time 0 and at 5, 20, and 28 h post-wounding using the $\times 10$ objective of an inverted phase-contrast microscope. The line tool in Canvas 9 (ACDSee Systems) 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) were evaluated in BG1 and HEY ovarian cancer cells. In brief, 2×10^4 cells that were transfected with control or YY1-specific siRNA oligos were treated with each drug for 24 h beginning 5 h post-transfection. The effect of treatment was measured using the

CellTiter 96 AQueous One Solution Cell Proliferation Assay as described above. IC_{50} values were calculated for each data point using GraphPad Prism 4 software (GraphPad Software). Knockdown experiments, followed by chemosensitivity assays, were repeated 10 times.

Statistical Analysis

For ranking of genes with or without TRANSFAC motifs, the Mann-Whitney *U* test was done. Ranking of genes with or without the GO cellular component annotation of microtubule was also analyzed using the Mann-Whitney *U* test and χ^2 tests where appropriate. Survival comparison was done using a log-rank test. Paired *t* tests were used to compare IC_{50} values between control and YY1 siRNA-treated samples. Deviation of borderline tumors among clusters was calculated using Fisher's exact test. IC_{50} value differences between clusters in the NCI60 data set were calculated using Mann-Whitney *U* tests. For these calculations, GraphPad Prism 4.0b software was used. The enrichment of GO terms for "biological process" was analyzed using Gene Annotation Tool to Help Explain Relationships (21). For other statistical analyses, two-tailed Student's *t* tests were used. *P* values < 0.05 were considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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