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Oncogenic Ras and B-Raf Proteins Positively Regulate Death Receptor 5 Expression through Co-activation of ERK and JNK Signaling*[S]

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Background: The oncogene Ras induces *DR5* expression with undefined mechanism.
Results: Both Ras and B-Raf induce *DR5* expression through co-activation of ERK and JNK signaling and subsequent cooperative effects among CHOP, Elk1, and c-Jun.
Conclusion: Co-activation of ERK and JNK signaling accounts for Ras-induced *DR5* expression.
Significance: A novel function of DR5 in Ras- or B-Raf-mediated oncogenesis may be suggested.

Oncogenic mutations of *ras* and B-*raf* frequently occur in many cancer types and are critical for cell transformation and tumorigenesis. Death receptor 5 (DR5) is a cell surface pro-apoptotic death receptor for tumor necrosis factor-related apoptosis-inducing ligand and has been targeted in cancer therapy. The current study has demonstrated induction of *DR5* expression by the oncogenic proteins Ras and B-Raf and revealed the underlying mechanisms. We demonstrated that both Ras and B-Raf induce *DR5* expression by enforced expression of oncogenic Ras (*e.g.* H-Ras12V or K-Ras12V) or B-Raf (*i.e.* V600E) in cells and by analyzing gene expression array data generated from cancer cell lines and from human cancer tissues. This finding is further supported by our results that knockdown of endogenous K-Ras or B-Raf (V600E) reduced the expression of *DR5*. Importantly, we have elucidated that Ras induces *DR5* expression through co-activation of ERK/RSK and JNK signaling pathways and subsequent cooperative effects among the transcriptional factors CHOP, Elk1, and c-Jun to enhance *DR5* gene transcription. Moreover, we found that the majority of cancer cell lines highly sensitive to the DR5 agonistic antibody AMG655 have either Ras or B-Raf mutations. Our findings warrant further study on the biology of DR5 regulation by Ras and B-Raf, which may provide new insight into the biology of Ras and B-Raf, and on the potential impact of Ras or B-Raf mutations on the outcome of DR5-targeted cancer therapy.

Ras proteins, including H-Ras, K-Ras, and N-Ras, share similar structure and function and can become constitutively activated by mutation. Mutant Ras proteins are resistant to down-regulation by GAP-mediated hydrolysis of bound GTP and, therefore, signal persistently (1). It has been documented that activating mutations in *ras* genes are present in 15% of all cancers and perhaps as many as 30% of metastatic human cancers (2). The mutant Ras proteins typically activate the Raf/MEK/extracellular signal-regulated kinase (ERK) kinase cascade, which is often associated with the promotion of cell proliferation, and the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, which functions to suppress apoptosis and contributes to oncogenic transformation (1, 3, 4).

Moreover, Ras has been suggested to promote apoptosis. One mechanism accounting for this process involves the association of activated Ras with a Nore1-RASSF1-Mst1 complex (4, 5). In addition, it has been shown that protein kinase C-mediated phosphorylation of the K-Ras membrane-anchoring domain can trigger K-Ras release from the plasma membrane and relocation onto the outer mitochondrial membrane to interact with Bcl-X_L, resulting in induction of apoptosis (6, 7). This apoptosis-inducing activity of Ras may exert a suppressive effect on Ras-induced oncogenesis by preventing survival of transformed cells.

Death receptor 5 (DR5[3]; also called TRAIL-R2 or killer/DR5) is one of the death domain-containing cell surface receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a tumor-selective apoptosis-inducing cytokine with potential as a cancer therapeutic agent. When overexpressed or ligated with its ligand, TRAIL, DR5 becomes oligomerized (trimerized) and rapidly activates the extrinsic apoptotic pathway. This process involves trimerized DR5 interacting specifically with the adaptor protein Fas-associated death domain via death domain interaction and subsequent recruitment of caspase-8 through the death effector domain between Fas-as-

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[3] The abbreviations used are: DR5, death receptor 5; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; RSK, ribosomal S6 kinase.

sociated death domain and caspase-8, leading to caspase-8 activation and ultimately, apoptosis (8).

DR5 expression can be induced by enhancing its transcription. The transcriptional factors p53 (9, 10), NF-κB (11, 12), C/EBP homologous protein (CHOP; also known as growth arrest and DNA damage-inducible protein 153 (GADD153)) (13, 14), Elk1 (15), and YY1 (16) have been suggested to be involved in this process. Interestingly, the oncogenic Ras was previously shown to induce *DR5* expression and to sensitize cells to TRAIL-induced apoptosis (17, 18). However, the detailed mechanism underlying Ras-induced *DR5* expression has not been elucidated.

The Raf/MEK/ERK kinase cascade represents the predominant and best studied effector pathway downstream of Ras and is critical for Ras-induced oncogenesis (1, 4). The Raf proteins, including A-Raf, B-Raf, and C-Raf/Raf-1, are a family of serine/threonine kinases and can bind to and are activated by GTP-bound Ras. Raf activation results in activation of the MAPK cascade through phosphorylation of MEK which, in turn, phosphorylates ERK. Following phosphorylation, ERK translocates to the nucleus where it activates various transcription factors or directly phosphorylates 90-kDa ribosomal S6 kinase (RSK), another conserved serine/threonine kinase, which can also translocate to the nucleus and activates transcription through direct phosphorylation (1, 19, 20). Activation of Raf/MEK/ERK signaling is generally associated with stimulation of cell proliferation, including promoting cell survival by suppression of apoptosis; however, a growing number of studies also suggest that activation of this signaling pathway can promote cell death, including apoptosis (19, 21–23).

In this study, we further studied Ras-induced *DR5* expression in a comprehensive way by enforced expression of oncogenic Ras in cells and by analyzing gene expression array data generated from cancer cell lines and from human cancer tissues. Moreover, we also demonstrated for the first time that oncogenic B-Raf induces *DR5* expression. Most importantly, we have revealed the mechanisms by which Ras or B-Raf induces *DR5* expression. Our results show that the cooperative interaction between CHOP, Elk1, and c-Jun activated by the ERK/RSK and c-Jun N-terminal kinase (JNK) signaling pathways mediates Ras-induced *DR5* expression.

EXPERIMENTAL PROCEDURES

Reagents and Cell Lines—Rabbit HA tag antibody was purchased from Abgent (catalog no. AP1012, San Diego, CA). Mouse Anti-Ras antibody (clone RAS10; catalog no. 05-512) was purchased from Millipore (Billerica, MA). B-Raf antibody was purchased from Cell Signaling Technology (Beverly, MA). Other antibodies were the same as described previously (15). The human monoclonal DR5 agonistic antibody, AMG655 (Conatumumab), was supplied by Amgen (Thousand Oaks, CA). HEK-293T cells were provided by K. Ye (Emory University, Atlanta, GA). The human lung cancer cell lines A549, H1792, H157, and Calu-1 were described previously (24). The rest of the cancer cell lines were provided by Dr. P. Giannakakou (Weill Medical College of Cornell University, New York, NY).

Expression Constructs and Transfection—*CHOP* and *Elk1* expression constructs were described previously (15). c-*jun* expression plasmid (pCMV-c-Jun) was obtained from Dr. M. J. Birrer (NCI, NIH) and used in our previous study (25). HA-tagged human wild-type (WT) and constitutively activated (G12V) and dominant-negative (S17N) H-*ras* expression plasmids in pcDNA3.1 vector were obtained from UMR cDNA Resource Center (Rolla, MO) and provided by Dr. F. J. Shu (Emory University). WT EGFP-K-*ras* or B-*raf* constructs were obtained from Dr. H. Fu (Emory University). Mutant K-*ras* (12V, 12C, 12D, or 13D) and B-*raf* (V600E) expression constructs were generated by site-directed mutagenesis method using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Cell transfection with the given plasmids was conducted using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol.

Western Blot Analysis—Whole cell protein lysates were prepared and analyzed by Western blotting as described previously (26).

Reporter Plasmids and Luciferase Activity Assay—All *DR5* reporter constructs used in this study were described previously (15, 27). Plasmid transfection and luciferase assays were the same as described previously (27).

Gene Silencing Using siRNA—Gene silencing was achieved by transfecting siRNA using HiPerFect transfection reagent (Qiagen, Valencia, CA) following the manufacturer's instructions. Control (*i.e.* non-silencing), CHOP, Elk1, and ERK1/2 siRNA and RSK2 shRNA were described previously (15, 27). shRSK1 (RHS3979–9569862) was purchased from Open Biosystems (Huntsville, AL). c-Jun (#6205) and JNK (#6234) siRNAs were purchased from Cell Signaling Technology. K-Ras (for both WT and mutant genes) siRNA, which targets the sequence of 5′-AGCAAGTAGTAATTGATGGAG-3′, and B-Raf (V600E) siRNA, which targets the sequence of 5′-TCTAGCTACAGAGAAATCTCG-3′, were synthesized from Qiagen. Gene silencing effects were evaluated by Western blot analysis as described above.

Oligonucleotide Pulldown Assay—The WT and mutant oligonucleotides corresponding to the human *DR5* promoter region (−334 to −253) harboring Elk1 and CHOP binding sites, with biotin added to their 5′-ends were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The sequences for WT oligonucleotides were biotin-5′-CAGGGCGAAGGTTAGTTCCGGTCCCTTCCCCTCCCCTCCCCACTTGGACGCGCTTGCGGAGGATTGCGTTGACGAGACTCTT-3′ (DR5-wt, forward) and 5′-AAGAGTCTCGTCAACGCAATCCTCCGCAAGCGCGTCCAAGTGGGGAGGGGAGGGGAAGGGACCGGAACTAACCTTCGCCCTG-3′-biotin (DR5-wt, reverse). The sequences for mutated oligonucleotides in which both CHOP and Elk1 sites were altered were biotin-5′-CAGGGCGAAGGTTAGCTAGATTCACTTCCCCTCCCCTCCCCACTTGGACGCGCTTGCGGAGTACATAGTCGACGAGACTCTT-3′ (DR5-m, forward) and 5′-AAGAGTCTCGTCGACTATGTACTCCGCAAGCGCGTCCAAGTGGGGAGGGGAGGGGAAGTGAATCTAGCTAACCTTCGCCCTG-Biotin-3′ (DR5-m, reverse). Each pair of oligonucleotides was annealed following standard protocols. The oligonucleotide pulldown assay was carried out as described previously (28). In brief,

500 μl of whole cell protein lysate was mixed with biotinylated double-stranded oligonucleotides and ImmunoPure streptavidin-agarose beads (Thermo Scientific/Pierce, Rockford, IL). The mixture was incubated at 4 °C for 16 h with shaking. The beads were then pelleted and washed with cold lysis buffer for four times. Bound proteins were finally separated by SDS-PAGE followed by Western blot analysis to detect proteins of interest with specific antibodies.

Cell Survival Assay—Cells were seeded in 96-well cell culture plates and treated the next day with the tested agent. The viable cell number was determined using sulforhodamine B assay as described previously (29).

Data Source for Analysis of ras and B-raf Mutation and DR5 Expression in Cancer Cell Lines—The NCI-60 U133 microarray data on *DR5* (*i.e.* TNFRSF10B) expression (including probesets 209295_at, 209292_x_at, and 210405_x_at) were obtained from National Institutes of Health/NCI Cancer Genome Anatomy Project (available on-line at cgap.nci.nih.gov/Microarray/MicroarrayAccessions?ORG=Hs&CID=521456). The *ras* (including K-*ras* and H-*ras*) and B-*raf* mutation information on the analyzed cancer cell lines was obtained from the Wellcome Trust Sanger Institute Cancer Genome Project (www.sanger.ac.uk/perl/genetics/CGP/core_line_viewer?action=nci60_list). *DR5* expression in Affymetrix U133 + 2.0 microarray data from human mammary epithelial cells infected with adenovirus expressing either human c-Myc, activated H-Ras, human c-Src, human E2F3, or activated β-catenin were extracted from the Gene Expression Omnibus (GSE3151) (30). *DR5* expression in Affymetrix U133A microarray data from cancer cell lines with and without B-*raf* V600E mutation before and after an 8-h treatment with the MEK1/2 inhibitor PD325901 (31) were extracted from the Gene Expression Omnibus (GSE10086).

Data Source for Analysis of K-ras Mutation and DR5 Expression in Human Cancer Tissues—Microarray data from 80 metastatic colorectal cancer patients were downloaded in series matrix format from Gene Expression Omnibus record GSE5851. The data were log2-transformed, and probesets corresponding to *DR5* were extracted (209295_at, 209294_x_at, and 210405_x_at). The highest signal intensities were associated with probeset 209295_at, whereas probeset 210405_x_at did not yield high signal intensity in any sample. The K-*ras* gene mutational status corresponding to these samples was extracted from the study published by Khambata-Ford *et al.* (32).

Statistical Analysis—The statistical significance of differences in *DR5* expression between the two groups was analyzed with two-sided unpaired Student's *t* tests or Fisher's exact test by use of InStat 3 software (GraphPad, San Diego, CA). Results were considered to be statistically significant at $p < 0.05$.

RESULTS

Oncogenic Ras and B-Raf Activate DR5 Promoter and Induce DR5 Expression—We first examined the effects of H-Ras on *DR5* transcriptional activity and expression. To this end, we co-transfected WT or mutant H-*ras* gene with a *DR5* luciferase reporter construct carrying −522 bp of the *DR5* promoter region and then analyzed luciferase activity. As presented in Fig. 1*A*, enforced expression of the WT and constitutively active mutant (12V) H-Ras, but not dominant-negative mutant (S17N) H-Ras, increased *DR5* promoter activity. However, H-Ras12V induced the greatest increase in *DR5* promoter activity. In agreement, enforced expression of H-Ras12V, but not H-RasS17N, substantially increased DR5 protein levels (Fig. 1*B*). Time course analysis showed that H-Ras12V expression was detected at 36 h post-transfection and sustained up to 60 h. In parallel, *DR5* induction was observed within the same time range (Fig. 1*C*). We also analyzed *DR5* expression in publicly available gene expression array data generated from human mammary epithelial cells following adenovirally enforced expression of H-Ras, E2F3, c-Myc, c-Src, or activated β-catenin, with GFP as a control (30). As presented in supplemental Fig. S1, only infection with activated Ras appreciably and significantly induced expression of *DR5* ($p < 0.0001$). These data taken together clearly indicate that oncogenic H-Ras induces *DR5* expression.

Furthermore, we analyzed the effect of the oncogenic K-Ras12V on *DR5* expression and found that its enforced expression increased *DR5* promoter activity and *DR5* expression (supplemental Fig. S2, *A* and *B*). Similar to K-Ras12V, other cancer-derived K-Ras mutants, including 12C, 12D, and 13D, could also transactivate the *DR5* promoter and elevate *DR5* expression (supplemental Fig. S2, *C* and *D*).

Because the activation of the ERK/RSK signaling mediates *DR5* expression (15), we pondered whether the constitutively activated B-Raf mutant (V600E) would also induce *DR5* expression. Therefore, we further analyzed the effect of B-Raf (V600E) on *DR5* promoter activity and expression. Indeed, enforced expression of B-*raf* (V600E) increased both *DR5* promoter activity and *DR5* expression (Fig. 2, *A* and *B*). Therefore, not only H-Ras, but also K-Ras and B-Raf, up-regulate *DR5* expression.

To further demonstrate whether *DR5* expression is regulated by endogenous Ras or B-Raf, we used an siRNA approach to knock down K-Ras or mutant B-Raf (V600E) in a few cancer cell lines with K-*ras* (A549, H1792, H157, Calu-1, HCT15, and HCT116) or B-*raf* (V600E) (HT29, BCPAP, and LOXIMVI) mutation. As presented in Figs. 1*D* and 2*C*, knockdown of K-Ras or B-Raf (V600E) decreased the levels of p-ERK1/2 accompanied with reduction of *DR5* expression in these cell lines, indicating that the Ras/B-*raf* signaling indeed positively regulates endogenous *DR5* expression. These data provide further strong support for Ras and B-Raf regulation of *DR5* expression.

Cancer Cell Lines and Tissues with ras and/or B-raf Mutations Exhibit Elevated DR5 Expression—To further explore the association between *ras* or B-*raf* mutations and *DR5* expression, we asked whether *DR5* expression is in general higher in cancer cell lines or cancer tissues with *ras* or B-*raf* mutations than those without these mutations. We took advantage of available published data warehoused in public domains and databases for this analysis. We first analyzed *DR5* expression levels in microarray data generated from certain cancer cell lines that represent four types of cancers with high frequencies of K-*ras* and/or B-*raf* mutations (melanoma, colon cancer, non-small cell lung cancer, and breast cancer) (33), among the NCI-60 cancer cell line panel (NCI60_U133). The highest sig-

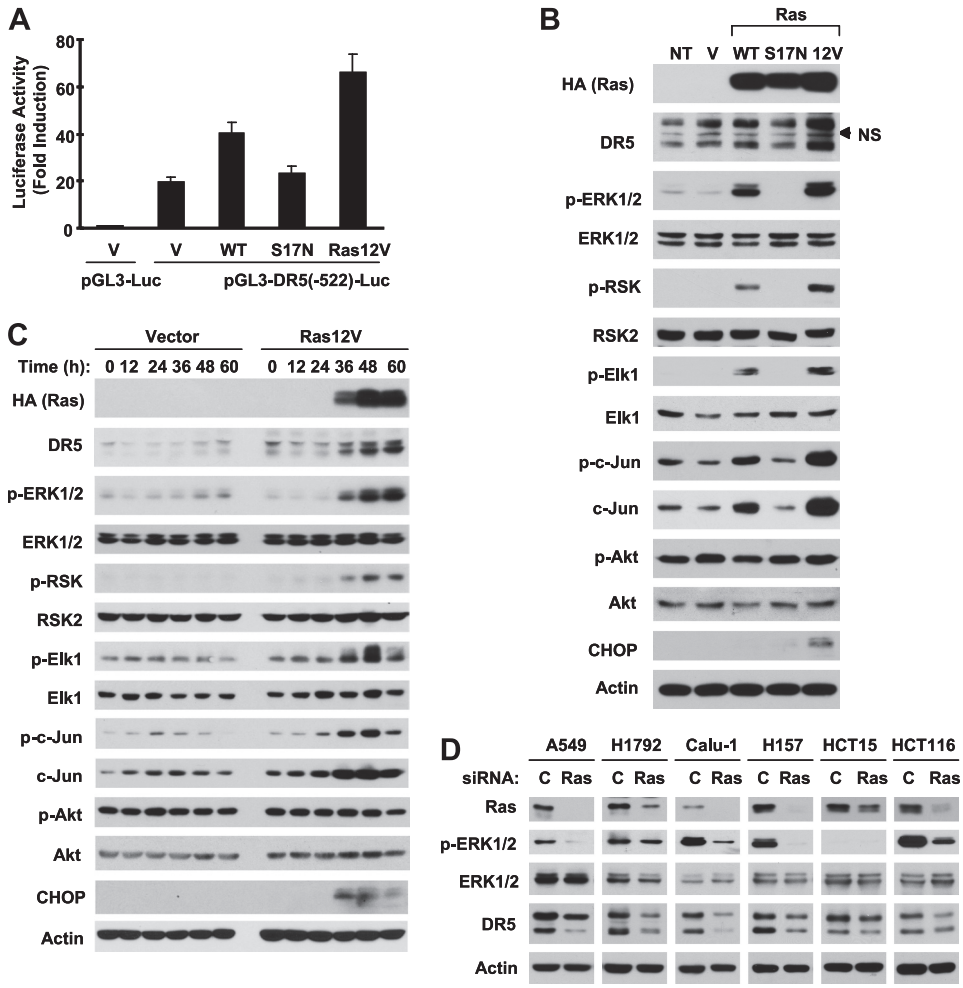

FIGURE 1. **Activated H-Ras transactivates the *DR5* promoter (*A*) and induces *DR5* expression along with activation of ERK/RSK and JNK signaling pathways and up-regulation of CHOP (*B* and *C*), whereas knockdown of K-Ras reduces *DR5* expression along with ERK inhibition (*D*).** *A*, HEK293T cells were co-transfected with *DR5* promoter reporter plasmid and the indicated H-*ras* genes. After 36 h, the cells were harvested for measurement of luciferase activity. The data are means ± S.D. of triplicate determinations. *B* and *C*, HEK293T cells were transfected with the expression plasmids carrying the given H-*ras* genes for 40 h (*B*) or the indicated times (*C*). *D*, the indicated cell lines with mutant K-*ras* were transfected with control or K-Ras (Ras) siRNA for 62 h. After the aforementioned transfections, the cells were harvested for preparation of whole cell protein lysates and subsequent Western blotting to detect the indicated proteins. *NT*, no transfection; *V*, vector; *NS*, nonspecific band; *C*, control.

nal intensities in the microarray analyses were generated with probeset 209295_at in comparison with signals generated with two other probesets (210405_x_at and 209294_x_at). Thus we first compared *DR5* expression between cancer cell lines with and without *ras* (*i.e.* K-*ras* and H-*ras*) and/or B-*raf* mutations. There were 21 cell lines with either K-*ras* (or H-*ras*) or B-*raf* mutation and 10 cell lines with WT genes among the cell lines analyzed. *DR5* mRNA levels were significantly higher in cell lines with mutant Ras or B-Raf than those with WT genes ($p = 0.0073$) (supplemental Fig. S3*A*). We used an arbitrary cutoff value of 150 to define "high" *DR5* expression and observed that 81% of cell lines (17/21) with *ras* or B-*raf* mutation expressed a high level of DR5 (*i.e.* >150). In contrast, only 30% (3/10) of cell lines without these mutations expressed a high level of DR5 (supplemental Fig. S3*D*). The difference in *DR5* expression between these two groups of cell lines was statistically significant ($p = 0.0135$). We also analyzed *DR5* expression data generated with probesets 209294_x_at and 210405_x_at. *DR5* expression levels with the probeset 210405_x_at were also significantly higher in cell lines with mutant *ras* or B-*raf* than those without these mutations ($p = 0.008$) (supplemental Fig. S3*B*). The microarray data with the probeset 209294_x_at showed a trend toward elevated DR5 in cell lines with mutant *ras* or B-*raf* compared with those without the mutations, although the difference was not statistically significant ($p = 0.0598$) (supplemental Fig. S3*C*). Collectively, these data clearly indicate that cancer cell lines harboring *ras* or B-*raf* mutation have elevated *DR5* expression.

Moreover, we extracted gene expression data and K-*ras* mutational status from a previously published cohort of 80 microarrays generated using metastatic tumor deposit in the liver from colorectal cancer patients (32) to test whether *DR5* gene expression is associated with K-*ras* mutational status in human cancer tissues. We found that *DR5* expression in K-*ras* mutant tumors was significantly higher than in those with WT K-*ras* when comparing the data generated from probeset 209295_at ($p = 0.005$) (supplemental Fig. S4*A*), further demonstrating a strong association between K-*ras* mutation or activation and high *DR5* expression in human cancer tissues. A similar trend was observed using data generated with the probeset

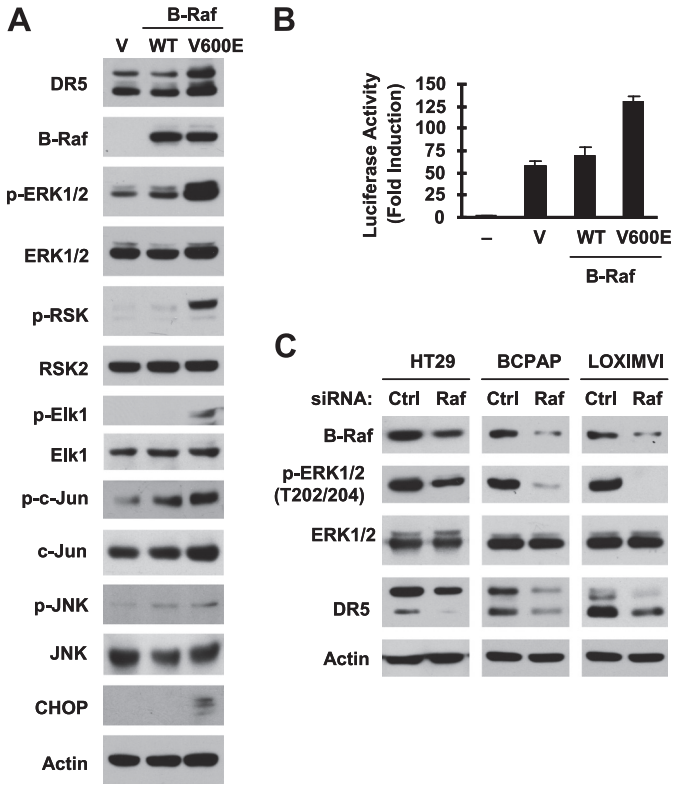

FIGURE 2. **Activated B-Raf transactivates the *DR5* promoter (*B*) and induces *DR5* expression along with activation of ERK/RSK and JNK signaling pathways and up-regulation of CHOP (*A*), whereas knockdown of B-Raf (V600E) reduces *DR5* expression along with ERK inhibition (*C*).** *A*, HEK293T cells were transfected with vector (*V*) or the indicated B-*raf* genes. After 40 h, the cells were subjected to preparation of whole cell protein lysates and subsequent Western blotting to detect the given proteins. *B*, HEK293T cells were co-transfected with *DR5* promoter reporter plasmid and vector or the indicated B-*raf* genes. After 40 h, the cells were harvested for measurement of luciferase activity. Each *column* shows mean ± S.D. of triplicate determinations. *C*, the indicated cell lines with mutant B-*raf* were transfected with control (*Ctrl*) or B-*raf* (V600E) (*Raf*) siRNA for 62 h. The cells were then harvested for preparation of whole cell protein lysates and subsequent Western blotting to detect the indicated proteins.

209294_x_at, although this was not statistically significant ($p = 0.0629$) (supplemental Fig. S4*B*). Based on the "x_at" probeset designation, this probeset is known to target other gene transcripts as well. Therefore, the data from 209295_at are likely the most reliable. Collectively, these data strongly support our notion that activated Ras signaling, including B-Raf, positively regulates *DR5* expression.

Oncogenic Ras Activates the ERK/RSK and JNK Signaling Pathways and Their Regulated Proteins—To understand the mechanisms by which activated Ras induces *DR5* expression, we next examined the effect of Ras activation on ERK/RSK signaling and its regulated proteins. As presented in Fig. 1 (*B* and *C*), enforced expression of H-Ras, particularly H-Ras12V, increased the levels of p-ERK1/2, p-RSK, and p-Elk1, which paralleled H-Ras expression and DR5 induction. Thus, the activated H-Ras clearly activates ERK/RSK signaling in the tested cell system. Interestingly, we did not detect increased levels of p-Akt in cells transfected with either WT H-*ras* gene or mutant H-*ras*12V gene (Fig. 1, *B* and *C*), suggesting that H-Ras activation does not turn on PI3K/Akt signaling under the tested experimental conditions. However, we observed increased levels of both p-c-Jun and c-Jun in cells transfected with H-*ras*, particularly H-*ras*12V (Fig. 1, *B* and *C*). Thus, oncogenic H-Ras also activates JNK/c-Jun signaling. Similarly, enforced expression of oncogenic K-*ras*12V or B-*raf* (V600E) increased the levels of p-ERK1/2, p-RSK, p-Elk1, p-JNK, and p-c-Jun (Fig. 2*A* and supplemental Fig. S2*A*) (34) indicating that activated K-Ras and B-Raf also activate both the ERK/RSK and JNK signaling pathways in the tested cell system.

Oncogenic Ras Induces DR5 Expression through an ERK/RSK-dependent Mechanism—We next determined whether the activation of ERK and RSK signaling is required for Ras-induced *DR5* expression. To this end, we knocked down ERK1/2 expression with ERK1/2 siRNA to suppress Ras-induced ERK/RSK activation and then analyzed its impact on Ras-induced *DR5* expression. As shown in Fig. 3*A*, transfection of ERK1/2 siRNA not only reduced the levels of total ERK1/2 but also the levels of p-ERK, p-RSK, and p-Elk1 induced by H-Ras12V compared with the control siRNA, indicating successful knockdown and blockage of Ras-induced activation of ERK/RSK signaling. We detected increased levels of DR5 in control siRNA-transfected but not in ERK1/2 siRNA-transfected cells (Fig. 3*A*). In agreement, we observed that H-*ras*12V increased *DR5* promoter activity in cells transfected with the control siRNA but not in cells transfected with ERK1/2 siRNA (Fig. 3*B*). Moreover, we determined whether RSK activation is also involved in mediating Ras-induced *DR5* expression. Expression of either shRSK2 or shRSK1 substantially reduced the levels of RSK2 or RSK1, respectively, indicating the successful knockdown of their expression. Enforced expression of H-Ras12V increased *DR5* expression in the vector control cells (pLKO1) but failed to do so in cells expressing shRSK2 or shRSK1 (Fig. 3*C*), indicating that both RSK2 and RSK1 are involved in mediating Ras-induced *DR5* expression. Similarly, H-Ras12V increased *DR5* promoter activity in pLKO1-transfected cells but only minimally in cells transfected with either shRSK1 or shRSK2 (Fig. 3*D*). Collectively, these data clearly demonstrate that oncogenic Ras induces *DR5* expression in an ERK/RSK-dependent manner.

The above findings led us to further analyze whether this paradigm held true in cancer cell lines harboring activated B-Raf (V600E) following chemical MEK inhibition with the compound PD325901. For this analysis, we extracted *DR5* expression data from publicly available microarray data reported by Pratilas *et al.* (31). Consistent with the above-discussed data, B-Raf V600E cell lines demonstrated higher baseline expression of *DR5* than B-Raf WT cell lines. MEK inhibition with PD325901 (8 h) significantly reduced *DR5* gene expression in B-Raf V600E cell lines ($p = 0.009$), but not in B-Raf WT cell lines ($p > 0.05$) (supplemental Fig. S5). These data provide further support for the critical role of the MEK/ERK signaling in mediating *DR5* expression induced by activated Ras or B-Raf.

Oncogenic Ras Also Induces a JNK-dependent DR5 Expression—Our previous studies suggest that JNK activation contributes to drug-induced DR5 up-regulation (35, 36). Because Ras activates JNK signaling in parallel with the activation of ERK/RSK signaling, we further asked whether JNK signaling is involved in Ras-induced *DR5* expression. To address this question, we inhibited

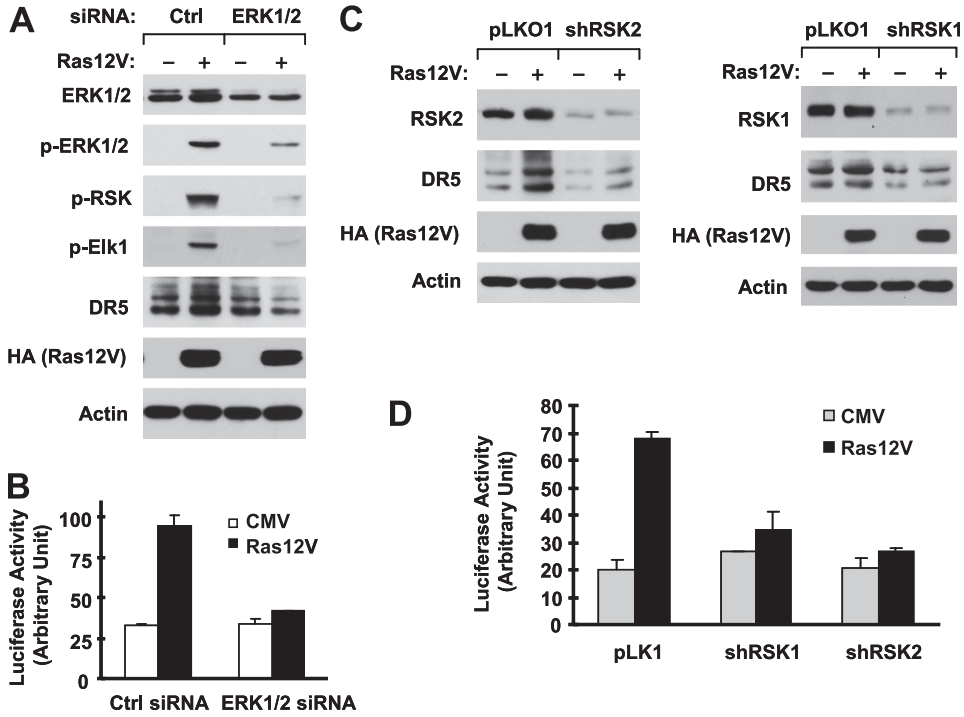

FIGURE 3. **Knockdown of ERK1/2 (*A* and *B*) or RSK (*C* and *D*) abolishes Ras-induced *DR5* expression (*A* and *C*) and promoter transactivation (*B* and *D*).** *A* and *B*, HEK293 cells were transfected with control (*Ctrl*) or ERK1/2 siRNA, and 15 h later, were transfected with vector or H-*ras*12V. After 40 h, the cells were harvested for preparation of whole cell protein lysates and subsequent Western blotting for the indicated proteins (*A*) or were lyzed for luciferase activity assay (*B*). Each *column* in *B* represents the mean ± S.D. of triplicate determinations. *C* and *D*, HEK293 cells expressing pLKO1, shRSK2, or shRSK1 were transfected with vector or H-*ras*12V. After 48 h, the cells were harvested for preparation of whole cell protein lysates and subsequent Western blotting (*C*) or were lysed for luciferase activity assay (*D*). Each *column* in *D* represents the mean ± S.E. of two experiments.

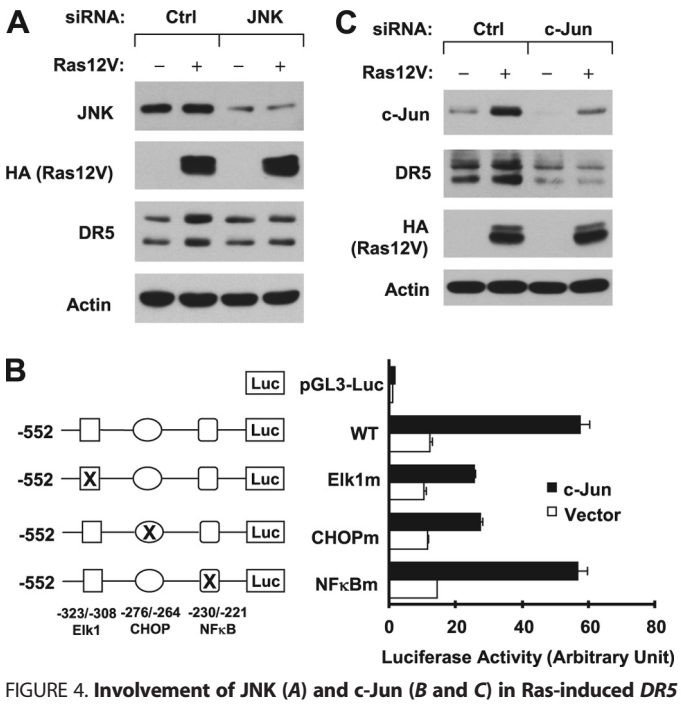

FIGURE 4. **Involvement of JNK (*A*) and c-Jun (*B* and *C*) in Ras-induced *DR5* expression.** *A*, HEK293 cells were transfected with control (*Ctrl*) or JNK siRNA, and 15 h later, were transfected with vector or H-*ras*12V. After 40 h, the cells were harvested for preparation of whole cell protein lysates and subsequent Western blotting for the indicated proteins. *B*, HEK293T cells were co-transfected with the indicated reporter constructs and vector or H-*ras*12V plasmids. After 42 h, the cells were lysed for luciferase activity assay. *C*, HEK293 cells were transfected with control (*Ctrl*) or c-Jun siRNA, and 15 h later, were transfected with vector or H-*ras*12V. After 40 h, the cells were harvested for preparation of whole cell protein lysates and subsequent Western blotting for the indicated proteins. The data are means ± S.D. of triplicate determinations.

JNK activation by knocking down JNK expression and then looked at its impact on Ras-induced *DR5* expression. As shown in Fig. 4*A*, transfection of JNK siRNA drastically reduced the levels of JNK, confirming its successful knockdown. Correspondingly, we detected an increase in *DR5* expression induced by H-*ras*12V transfection in control siRNA-transfected cells, but not in JNK siRNA-transfected cells, indicating that the activation of JNK signaling is also required for Ras-induced *DR5* expression.

The Transcriptional Factors, CHOP, Elk1, and c-Jun, Are Involved in Mediating Ras-induced DR5 Expression—We have recently shown that CHOP and Elk1 cooperate to mediate ERK/RSK-dependent DR5 up-regulation induced by certain small molecules (15). Therefore, we determined whether CHOP and Elk1 are involved in Ras-induced *DR5* expression. By analyzing the promoter region of the *DR5* gene through deletion analysis, we found that the region between −373 bp and −240 bp was required for H-Ras12V to increase *DR5* transcription, because H-Ras12V could increase luciferase activity of reporter constructs carrying −373 and −522 bp *DR5* promoter regions but failed to do so in cells transfected with reporter constructs harboring −240, −140, and −120 bp *DR5* promoter regions (Fig. 5*A*). Because the region between −373 bp and −240 bp of the *DR5* promoter contains putative CHOP (−276/−264) and Elk1 (−323/308) binding sites, we further analyzed the impact of mutations in these binding sites on Ras-induced *DR5* transactivation. We included mutation of the NF-κB binding site (−236/−221) as a negative control in this analysis. Mutation of the NF-κB binding site did not affect Ras-

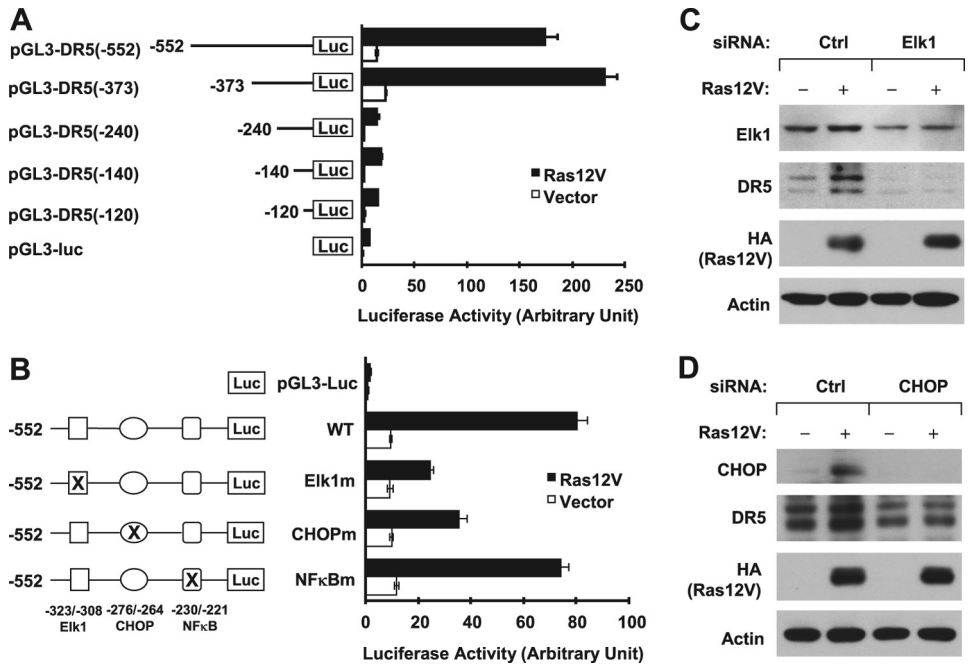

FIGURE 5. **Ras induces CHOP- and Elk1-dependent transactivation of *DR5* (*A* and *B*) and expression of *DR5* (*C* and *D*).** *A* and *B*, HEK293 cells were co-transfected with the indicated reporter constructs and vector or H-*ras*12V plasmids. After 40 h, the cells were lysed for luciferase activity assay. Each *column* represents the mean ± S.D. of triplicate determinations. *C* and *D*, HEK293 cells were transfected with control (*Ctrl*), Elk1 (*C*) or CHOP (*D*) siRNA, and 15 h later, were transfected with vector or H-*ras*12V. After 40 h, the cells were harvested for preparation of whole cell protein lysates and subsequent Western blotting for the indicated proteins.

induced *DR5* transactivation; however, mutation of either the CHOP or Elk1 binding site attenuated Ras-mediated *DR5* transactivation (Fig. 5*B*). These data suggest that both CHOP and Elk1 binding sites are important for Ras to transactivate the *DR5* gene, implying that both CHOP and Elk1 are involved in this process.

In the aforementioned *ras* or B-*raf* transfection experiments, increased CHOP was detected in cells transfected with H-Ras12V (Fig. 1), K-*ras*12V (supplemental Fig. S2), or B-*raf* (V600E) (Fig. 2) in addition to an increase in Elk1. To further demonstrate the involvement of CHOP and Elk1 in Ras-induced *DR5* expression, we knocked down Elk1 and CHOP expression individually, and then examined their impact on Ras-induced *DR5* expression. As presented in Fig. 5 (*C* and *D*), H-Ras12V increased *DR5* expression in control siRNA-transfected cells but not in cells transfected with either Elk1 siRNA (Fig. 5*C*) or CHOP siRNA (Fig. 5*D*). In agreement, the ability of H-Ras12V to increase *DR5* promoter activity was also impaired or attenuated in cells where Elk1 or CHOP expression was silenced by siRNA (supplemental Fig. S6). Together, these data demonstrate that both Elk1 and CHOP are indeed critical for mediating Ras-induced *DR5* expression.

Another transcriptional factor, c-Jun, was also up-regulated upon Ras activation in our cell system (Fig. 1 and supplemental Fig. S2). We explored whether c-Jun plays a role in regulation of *DR5* expression, including Ras-induced *DR5* expression. We first analyzed the effects of c-*jun* expression on transactivation of the *DR5* promoter. As presented in Fig. 4*B*, c-Jun indeed increased *DR5* promoter activity. Mutation of the NF-κB site did not affect the ability of c-Jun to transactivate the *DR5* gene; however, mutation of either the CHOP or Elk1 binding site attenuated its ability to increase *DR5* promoter activity. These data suggest that c-Jun transactivates the *DR5* gene through CHOP- and Elk1-mediated mechanisms. Furthermore, we knocked down c-Jun expression to block c-Jun up-regulation and then analyzed its effect on Ras-induced *DR5* expression. As shown in Fig. 4*C*, knockdown of c-Jun substantially suppressed the increase in c-*jun* expression induced by H-Ras12V. Consequently, knockdown of c-Jun not only reduced the basal levels of DR5, but it also abolished Ras-induced DR5 up-regulation. Hence, it is clear that c-Jun is also critical for Ras-induced *DR5* expression.

c-Jun Participates in CHOP- and Elk1-mediated Transcription of DR5 Gene—Because the *DR5* promoter region lacks an AP-1 binding site, we were interested in understanding how c-Jun contributes to Ras-induced *DR5* expression. It was previously shown that CHOP interacts with c-Jun (or AP-1) to regulate gene expression through binding to the AP-1 consensus binding sequence in AP-1 target genes (37, 38). Thus we considered that c-Jun may interact with CHOP and even Elk1 to enhance CHOP- and Elk1-mediated gene transcription (Fig. 6*A*). To test this hypothesis, we conducted an oligonucleotide pulldown assay to test whether c-Jun binds to CHOP and/or Elk1 in the *DR5* promoter. First, we determined whether the oligonucleotides with CHOP and Elk1 binding sites could pull down CHOP, Elk1, and c-Jun from protein lysates of cells co-transfected with CHOP, Elk1, and c-Jun expression plasmids in comparison with empty streptavidin beads (Fig. 6*B*). As presented in Fig. 6*C*, we could detect CHOP, Elk1, and c-Jun proteins in protein complexes pulled down by the oligonucleotides but not in protein complexes pulled down with the empty streptavidin beads (Fig. 6*C*). Moreover, we transfected H-*ras*12V into cells, and then incubated lysates with biotin-

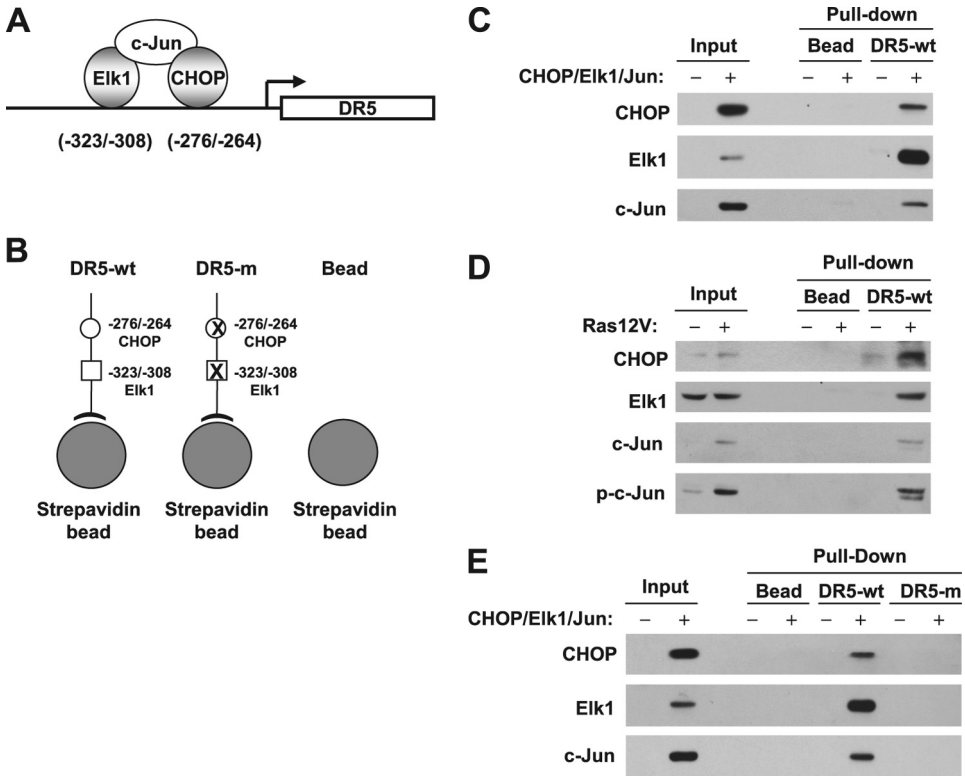

FIGURE 6. **Detection of CHOP, Elk1, and c-Jun bound to the *DR5* promoter region.** *A*, working model for regulation of *DR5* gene transactivation by CHOP, Elk1, and c-Jun. *B*, schematic illustration of biotin-labeled oligonucleotides used for studying DNA and protein interaction. *C* and *E*, HEK293T cells were co-transfected with CHOP, Elk1, and c-Jun expression plasmids for 48 h. *D*, HEK293T cells were transfected with empty vector or H-*ras*12V for 48 h. After the aforementioned treatments (*C–E*), whole cell protein lysates were then prepared from these cells and subjected to the oligonucleotide pulldown assay and subsequent Western blot analysis for the indicated proteins as described under "Materials and Methods." *wt*, wild-type; *m*, mutated.

labeled *DR5* oligonucleotides or streptavidin beads for the oligonucleotide pulldown assay. Again we detected CHOP, Elk1, c-Jun, and p-c-Jun only in the complexes pulled down with the *DR5* oligonucleotides from cell lysates expressing H-Ras12V (Fig. 6*D*). To robustly demonstrate this mechanism, we repeated the above experiments with a mutant oligonucleotide, in which both CHOP and Elk1 sites were mutated (Fig. 6*B*). Identical to the data presented in Fig. 6*C*, we found that the WT *DR5* oligonucleotides, but not the mutant *DR5* oligonucleotides or empty streptavidin beads, could pull down CHOP, Elk1, and c-Jun proteins from cells co-transfected with these three genes (Fig. 6*E*). Collectively, these data clearly suggest that c-Jun forms a complex with CHOP and/or Elk1 that binds to the *DR5* promoter.

Furthermore, we compared the effects of single, double, or triple transfection of CHOP, Elk1, and/or c-Jun on *DR5* gene transactivation and expression. The triple expression of CHOP, Elk1, and c-Jun induced the highest levels of *DR5* promoter activity and *DR5* expression compared with either single or double transfection (supplemental Fig. S7), suggesting that these three transcriptional factors cooperate to enhance *DR5* gene transcription.

Mutation Status of ras or B-raf Impacts Cell Response to DR5 Agonistic Antibody—Given that DR5 agonistic antibody exerts anticancer efficacy exclusively through binding to DR5 and inducing DR5-mediated apoptosis, we speculated that cancer cell lines with mutant *ras* or B-*raf* might be particularly susceptible to DR5-targeted cancer therapy due to elevated *DR5* expression. Thus, we examined the effects of AMG655, a human monoclonal DR5 agonistic antibody, on the survival of 38 human cancer cell lines, which consist of several cancer types known to have high frequency of *ras* or B-*raf* mutations (*e.g.* melanoma, colon, lung, breast, and pancreatic cancer). These cell lines showed varied sensitivities to AMG655. Among them there were 12 cell lines that were highly sensitive to AMG655 (IC_{50} values $<$ 300 ng/ml), 10 cell lines that were insensitive to AMG655 ($<$25% cell killing at 2000 ng/ml), and 16 cell lines showing intermediate sensitivity (IC_{50} values $\geq$ 1000 ng/ml) (Fig. 7*A* and supplemental Fig. S8). If we consider only the highly sensitive cell lines as responders, the response rate in cancer cell lines with *ras* or B-*raf* mutation was 44% (11/25), which was significantly higher than the 7.7% (1/13) in the cancer cell lines without these mutations ($p = 0.03$) (Fig. 7*B*). When we looked at the sensitive cancer cell line group alone, we noted that 91.7% (11/12) of the cancer cell lines had either a *ras* or B-*raf* mutation in comparison with only 8.3% (1/12) of cancer cell lines without these mutations (Fig. 7*C*). These data suggest that cancer cell lines with *ras* or B-*raf* mutation have a higher likelihood to respond to DR5 agonistic antibody than those without these mutations.

DISCUSSION

In addition to overexpression approaches, as conducted by other investigators previously (17, 18), the current study has further demonstrated Ras-mediated DR5 regulation under physiological conditions by knocking down endogenous K-*ras*

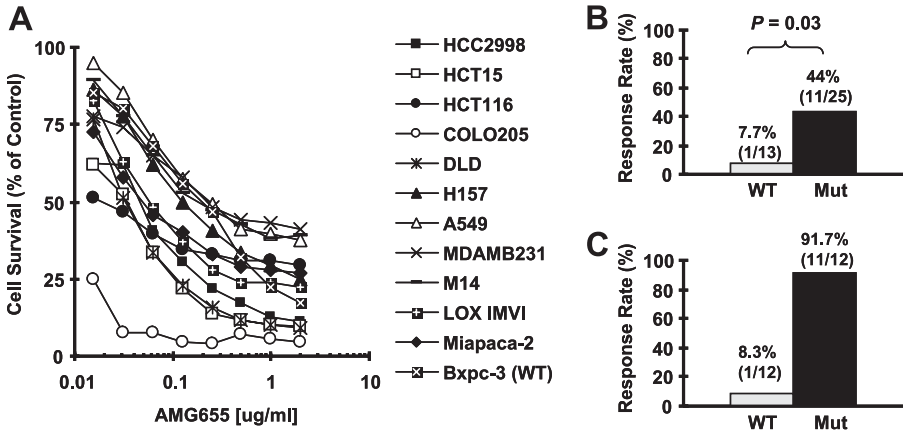

FIGURE 7. **Cancer cell lines with *ras* or B-*raf* mutation show high sensitivity to AMG655.** The given cell lines were seeded in 96-well plates and the next day treated with the indicated doses of AMG655 for 24 h. The number of viable cells was then estimated with the sulforhodamine B assay. The data represent the mean of four replicate determinations (*A*). S.D. values are <5% in general. Cancer cell lines with *ras* or B-*raf* mutation show significantly higher response rate to AMG655 than those without these mutations. The data between two groups were analyzed with Fisher's exact test (*B*). Among the highly sensitive cell lines to AMG655, the majority possess Ras or B-Raf mutation (*C*). Bxpc-3 is the only cell line with WT *ras* and B-*raf* genes.

expression in cancer cell lines (Fig. 1*D*). Thus, our current data provide robust evidence supporting that oncogenic Ras positively regulates *DR5* expression. Moreover, our analysis of *DR5* expression in a cohort of 80 metastatic human colon cancer tissues with known K-*ras* mutation status has shown significant elevation of *DR5* expression in tissues with K-*ras* mutation compared with those with WT K-*ras* (supplemental Fig. S4), providing the first *in vivo* evidence that *ras* mutations impact *DR5* expression in human cancer tissues.

Previous studies with chemical inhibitors suggested that MEK or ERK activation is involved in Ras-mediated DR5 up-regulation (17, 18). However, the precise mechanism by which Ras induces *DR5* expression was unknown. Using specific siRNA and shRNA approaches, we confirmed ERK-dependent DR5 induction by Ras, by demonstrating that inhibition of ERK signaling by knocking down ERK1/2 expression abrogated Ras-induced activation of *DR5* promoter and *DR5* expression (Fig. 3). Moreover, we further showed that knockdown of RSK2 or RSK1, well known ERK substrates, also suppressed Ras-induced *DR5* expression (Fig. 3). Thus, not only ERK- but also RSK-mediated signaling is involved in Ras-induced *DR5* expression.

Raf proteins as a family of serine/threonine kinases mediate Ras-induced ERK activation (1). In this study, we further showed that the oncogenic B-Raf (V600E), a commonly mutated form in cancers, activated ERK/RSK signaling, increased *DR5* promoter activity, and up-regulated *DR5* expression (Fig. 2). Analysis of *DR5* expression in publicly available microarray data also demonstrated that cancer cell lines with mutant B-*raf* (V600E) expressed significantly higher levels of DR5 in cell with WT B-*raf* (supplemental Fig. S5). In agreement, knockdown of mutant B-Raf (V600E) in cancer cells decreased *DR5* expression (Fig. 2). Moreover, chemical inhibition of MEK with PD325901 significantly reduced *DR5* expression levels only in cancer cell lines harboring the mutant B-*raf* gene (supplemental Fig. S5), indicating an MEK/ERK-dependent DR5 regulation by B-Raf. To our knowledge, this is the first study to show that B-Raf activation induces *DR5* expression.

In addition to activation of the Raf/MEK/ERK signaling cascade, oncogenic Ras also activates the PI3K/Akt and JNK/Jun signaling pathways (3). In our cell system, we did not detect increased p-Akt levels in H-*ras*12V-transfected cells (Fig. 1), suggesting that PI3K/Akt signaling was not activated by Ras under the tested conditions. However, we did see substantially increased levels of p-JNK and/or p-c-Jun in cells transfected with H-*ras*12V or K-*ras*12V (Fig. 1 and supplemental Fig. S2), indicating that oncogenic Ras turns on JNK/Jun signaling. Knockdown of JNK with JNK siRNA inhibited Ras-induced *DR5* expression (Fig. 4). Moreover, c-Jun transactivated the *DR5* promoter, and siRNA-mediated knockdown of c-Jun abolished the ability of Ras to induce *DR5* expression (Fig. 4). Together, these results indicate that JNK/c-Jun signaling is involved in mediating Ras-induced *DR5* expression.

JNK signaling has been implicated in the regulation of drug-induced *DR5* expression (35, 36). However, the underlying mechanism is unknown. *DR5* promoter analysis from the current study suggests that the region between −373 and −240 is responsible for Ras-induced transactivation of the *DR5* gene (Fig. 5). This region contains both CHOP and Elk1 binding sites, but it lacks an AP-1 binding site. Mutation of either the CHOP or Elk1 binding site attenuated the ability of Ras to transactivate the *DR5* gene (Fig. 5), demonstrating that both binding sites are responsible for Ras-induced *DR5* transactivation. Enforced expression of the oncogenic Ras (both H- and K-Ras) or B-Raf (V600E) increased the levels of p-Elk1 and CHOP along with DR5 induction (Figs. 1, 2, and supplemental Fig. S2). Moreover, knockdown of either Elk1 or CHOP expression inhibited Ras-induced *DR5* gene transactivation (supplemental Fig. S6) and expression (Fig. 5), indicating that both CHOP and Elk1 are indeed critical mediators for Ras-induced *DR5* expression. These results are consistent with our recent findings that CHOP and Elk1 cooperatively regulate *DR5* expression induced by small molecule drugs (15), thus reinforcing the notion that CHOP- and Elk1-mediated gene transcription is critical for *DR5* expression. The unaddressed question is the role of c-Jun in CHOP- and Elk1-mediated *DR5* expression.

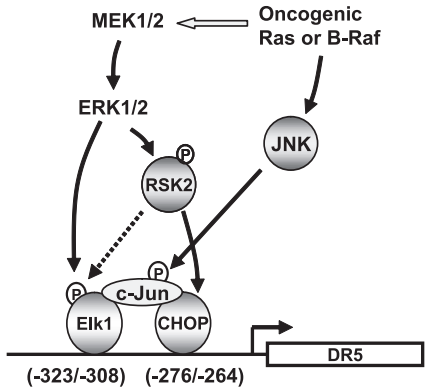

FIGURE 8. **Schematic working model for DR5 up-regulation induced by the oncogenic Ras or B-Raf.** This mechanism involves co-activation of the ERK/RSK- and JNK-signaling pathway via cooperative effect among CHOP, Elk1, and c-Jun to enhance *DR5* transcription.

It has been documented that CHOP interacts with c-Jun (or AP-1) to regulate gene expression through binding to the AP-1 consensus binding sequence in AP-1 target genes (37, 38). This led us to hypothesize whether c-Jun interacts with CHOP and even Elk1 and consequently enhances CHOP- and Elk1-mediated gene transcription. Indeed, c-Jun could be co-precipitated with CHOP and Elk1 in the oligonucleotide pulldown assay when these three proteins were co-expressed or when H-Ras was activated (Fig. 6), suggesting that c-Jun does form a complex with CHOP and/or Elk1 that binds to the *DR5* promoter region. Moreover, triple expression of CHOP, Elk1, and c-Jun induced the highest levels of *DR5* promoter activity and *DR5* expression compared with either single or double expression of these proteins (supplemental Fig. S7). Collectively these results demonstrate that c-Jun, likely as a co-activator, participates in CHOP- and Elk1-mediated *DR5* transcription through interaction with CHOP and/or Elk1 (Fig. 8).

We noted that the levels of *DR5* expression varied among different cell lines, although cancer cell lines harboring *ras* or B-*raf* mutation have significantly elevated *DR5* expression (supplemental Fig. S3). This variation is likely due to other regulatory mechanisms of *DR5* expression (*e.g.* p53, NF-κB, and YY1) (39).

It is well known that K-Ras mutation or activation is critical for cell transformation and cancer development, primarily through activation of the Raf/MEK/ERK and PI3K/Akt signaling pathways. However, Ras activation also leads to cell senescence and cell death (*e.g.* apoptosis), which may counteract its oncogenic function (4, 40–43). A recent mouse study has shown that deficiency of TRAIL receptor in mice (there is only one death receptor for TRAIL in mice) enhances metastasis without affecting primary tumor development (44), suggesting that TRAIL receptor or TRAIL-TRAIL receptor interaction may be critical for regulation of tumor metastasis. Thus, whether DR5 up-regulation plays a suppressive role in Ras-induced oncogenesis needs to be determined. Interestingly, accumulating evidence suggests that the presence or activation of death receptors (*e.g.* Fas) or other proteins in the death receptor pathways (*e.g.* caspase-8 and Fas-associated death domain) promotes tumor formation, growth, invasion, and even metastasis (45–52). Thus, it is also possible that Ras-induced *DR5* expression is involved in promoting Ras-mediated oncogenesis and/or metastasis, particularly under apoptosis-compromised conditions. Nonetheless, our current findings on Ras- or Raf-mediated DR5 up-regulation warrant further research in this direction.

DR5 agonistic antibodies have been widely tested as a novel type of cancer therapeutic in human clinical trials. However, the overall antitumor activity of these antibodies is modest or limited in unselected patient populations (53, 54). In this study, we found that cancer cell lines with *ras* or B-*raf* mutation showed a significantly higher response rate than those without these gene mutations (Fig. 7). This may imply that a given subtype of cancers with *ras* or B-*raf* mutations (*e.g.* colon cancer, lung cancer, thyroid cancer, or melanoma) may respond better to DR5 agonistic antibody-based therapies. Alternatively, K-*ras* or B-*raf* mutation may be a predictive biomarker for DR5-targeted cancer therapy. Thus, future study in this direction is warranted.

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