

Expression of Death Receptor 4 Is Positively Regulated by MEK/ERK/AP-1 Signaling and Suppressed upon MEK Inhibition*

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Death receptor 4 (DR4) is a cell surface receptor for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and triggers apoptosis upon ligation with TRAIL or aggregation. MEK/ERK signaling is a well known and the best-studied effector pathway downstream of Ras and Raf. This study focuses on determining the impact of pharmacological MEK inhibition on DR4 expression and elucidating the underlying mechanism. We found that several MEK inhibitors including MEK162, AZD6244, and PD0325901 effectively decreased DR4 protein levels including cell surface DR4 in different cancer cell lines. Accordingly, pre-treatment of TRAIL-sensitive cancer cell lines with a MEK inhibitor desensitized them to TRAIL-induced apoptosis. These results indicate that MEK inhibition negatively regulates DR4 expression and cell response to TRAIL-induced apoptosis. MEK inhibitors did not alter DR4 protein stability, rather decreased its mRNA levels, suggesting a transcriptional regulation. In contrast, enforced activation of MEK/ERK signaling by expressing ectopic B-Raf (V600E) or constitutively activated MEK1 (MEK1-CA) or MEK2 (MEK2-CA) activated ERK and increased DR4 expression; these effects were inhibited when a MEK inhibitor was present. Promoter analysis through deletion and mutation identified the AP-1 binding site as an essential response element for enhancing DR4 transactivation by MEK1-CA. Furthermore, inhibition of AP-1 by c-Jun knock-down abrogated the ability of MEK1-CA to increase DR4 promoter activity and DR4 expression. These results suggest an essential role of AP-1 in mediating MEK/ERK activation-induced DR4 expression. Our findings together highlight a previously undiscovered mechanism that positively regulates DR4 expression through activation of the MEK/ERK/AP-1 signaling pathway.

Death receptor 4 (DR4),⁴ also known as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor 1 (TRAIL-R1) or tumor necrosis factor receptor superfamily member 10A (TNFRSF10A), is a cell surface receptor that binds TRAIL and induces apoptosis. Similar to its sibling, death receptor 5 (DR5), TRAIL/DR4 ligation-induced apoptosis involves trimerized DR4 interacting specifically with the adaptor protein Fas-associated death domain (FADD) via death domain interaction. The subsequent recruitment of caspase-8 through the death effector domain of FADD leads to caspase-8 activation and ultimately, apoptosis (1, 2). In addition, agonistic DR4 antibody also induces DR4 aggregation, resulting in apoptosis through the same process (3, 4). For this reason, agonistic anti-DR4 antibodies have been developed as potential cancer therapeutics and tested in the clinic (5, 6).

In general, DR4 shares a redundant function with DR5 in mediating TRAIL-induced apoptosis. Thus, many agents, including some anticancer drugs, sensitize cancer cells to TRAIL-induced apoptosis through increasing the expression of DR4 and/or DR5 (7, 8). However, DR4 does display distinct functions from DR5, such as in mediating apoptosis induced by certain stimuli (9, 10) and in the regulation of cancer cell invasion and metastasis (11), although the underlying mechanisms are largely unknown. Like DR5, DR4 is also a p53 target gene and its expression can thus be regulated in a p53-dependent manner as we previously demonstrated (12, 13). Moreover, several p53-independent mechanisms that positively regulate DR4 expression including AP-1 (14), NF- κ B (15–17), c-Myc (18), and retinoic acid receptor (19)-mediated gene transcription have been suggested by us and others. Some agents increase DR4 expression through these mechanisms.

The MEK/ERK kinase cascade is a well known and the best-characterized effector pathway downstream of oncogenic RAS and RAF. This signaling pathway is often hyperactivated in many types of cancers, particularly those with RAS or RAF mutations such as melanoma, thyroid, and colon cancers, and hence plays a critical role in supporting the survival and proliferation of cancer cells. For the past decades, great effort has been devoted to developing effective anticancer drugs targeting

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⁴ The abbreviations used are: DR4, death receptor 4; DR5, death receptor 5; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MFI, mean fluorescence intensities; PARP, poly(ADP-ribose) polymerase; PE, phycoerythrin; MEK1-CA, constitutively activated MEK1; MEK2-CA, constitutively activated MEK2.

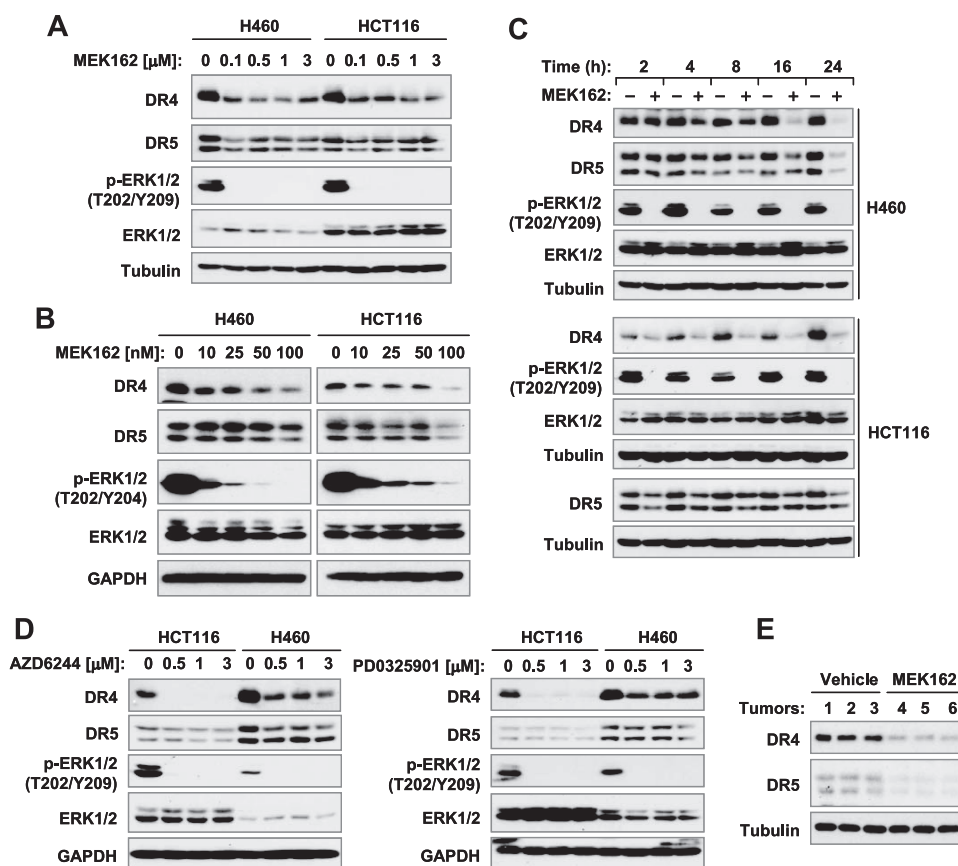


FIGURE 1. MEK inhibitors effectively suppress ERK phosphorylation and decrease the levels of DR4 and DR5 in cancer cell lines (A–D) and cancer xenografts (E). A–D, the indicated cancer cell lines were treated with different concentrations of MEK inhibitors as indicated for 13 h (A, B and D) or with 1 μ M MEK162 for the indicated times (C). The cells were then harvested for preparation of whole cell protein lysates and subsequent Western blotting analysis. E, whole protein cell lysates prepared randomly from 3 tumors treated with 5 mg/kg (oral gavage) of MEK162 for 21 days as conducted in our previous experiments were used for Western blotting analysis to detect the indicated proteins.

the RAS/RAF/MEK/ERK signaling pathway (20–23). The recent success of B-RAF and MEK inhibitors in the treatment of advanced melanoma represents a giant stride forward and has stimulated further research into the potential applications of this therapeutic strategy in other types of cancers (21, 22).

Although many agents increase *DR4* expression, some agents in fact decrease *DR4* expression through an unknown mechanism (24). While studying MEK inhibitors, we found that several of these agents substantially decrease the levels of DR4 accompanied with DR5 reduction in some cancer cell lines. Given our reported findings that RAS/RAF/MEK/ERK signaling positively regulates *DR5* expression through enhancing CHOP/Elk1-mediated gene transcription (11, 25, 26), we explored in this study whether the MEK/ERK signaling pathway also regulates *DR4* expression and investigated the underlying mechanism.

Results

MEK Inhibition with MEK Inhibitors Substantially Decreases DR4 Levels in Cancer Cells—While working with the MEK inhibitor, MEK162, we found that at the tested concentration ranges (1 and 3 μ M), it effectively decreased the levels of p-ERK1/2 in several lung cancer cell lines, indicating the potent inactivation of ERK1/2. Under such conditions, MEK162 not only decreased the levels of DR5, which is known to be posi-

tively regulated by MEK/ERK signaling (25–27) and used as a positive control here, but also drastically reduced DR4 levels in every cell line tested (data not shown). Similar results were generated in additional cancer cell lines, H460 (lung) and HCT116 (colon). Even at low concentration ranges up to 0.1 μ M, MEK162 effectively decreased DR4 levels accompanied with potent inhibition of ERK1/2 phosphorylation (Fig. 1A). We noted that at concentrations ranging from 0.1 to 3 μ M, the inhibition of p-ERK1/2, DR4, and DR5 by MEK162 was not concentration-dependent. Therefore we conducted another experiment with reduced concentration ranges (10 to 100 nM) of MEK162 in these two cell lines and found that MEK162 at this concentration range decreased p-ERK levels accompanied with DR4 reduction in a concentration-dependent manner (Fig. 1B). These data together suggest a good association between ERK inactivation and DR4 reduction. We noted that DR5 was reduced only at 100 nM, suggesting that DR4 is more susceptible than DR5 to modulation of the MEK/ERK signaling. We found that reduction of both DR4 and DR5 occurred early at 2 (HCT116) or 4 h (H460) and was sustained up to 24 h in cells post-MEK162 treatment (Fig. 1C). Here, we observed apparent ERK inhibition at 2 h in both tested cell lines after MEK162 treatment, suggesting that this is an earlier event than DR4 reduction. To determine whether other MEK inhibitors

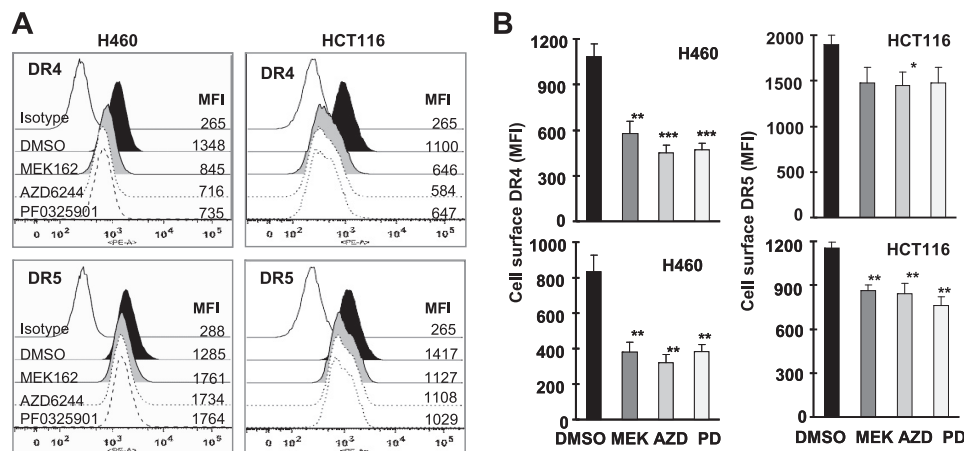


FIGURE 2. MEK inhibitors decrease cell surface DR4 and DR5 in cancer cells. Both H460 and HCT116 cells were treated with 1 μM MEK162, AZD6244, or PD0325901 for 12 h and then harvested for analysis of cell surface DR5 and DR4 by immunofluorescence staining and subsequent flow cytometry. The representative results are shown in A and average data from triplicate assays are presented in B as mean \pm S.D. The open peak in A represents DMSO-treated cells stained with a matched control PE-conjugated IgG isotype antibody. The filled black peaks show DMSO-treated cells stained with PE-conjugated anti-DR5 or DR4 antibody. The other peaks represent cells treated with different MEK inhibitors as indicated and stained with PE-conjugated anti-DR5 or DR4 antibody. MFIs for different treatments are indicated accordingly inside the graphs. *, $p < 0.017$; **, $p < 0.01$; ***, $p < 0.001$ compared with DMSO control.

have similar effects on decreasing DR4 and DR5, we treated H460 and HCT116 cells with AZD6244 and PD0325901 (0.5 to 3 μM) and found that both agents effectively reduced the levels of p-ERK1/2 and DR4 with relatively weak effects on decreasing DR5 levels (Fig. 1D). In xenograft tumors exposed to MEK162 for 2 weeks (28), we also observed reduction of DR5 and particularly DR4 (Fig. 1E), indicating a down-regulation of DR4 and DR5 by MEK inhibition *in vivo*. These data together strongly indicate that MEK inhibition with MEK inhibitors decreases DR4 expression, suggesting an association between MEK/ERK signaling and positive regulation of DR4 expression.

MEK Inhibitors Drastically Decrease Cell Surface DR4 Levels in Cancer Cells—Given that DR4 primarily functions on the cell membrane surface, we then used immunofluorescence staining and flow cytometry to detect the effects of MEK inhibitors on cell surface DR4 in comparison with cell surface DR5 in HCT116 and H460 cells. As presented in Fig. 2, the 3 tested MEK inhibitors, MEK162, AZD6244 and PD0325901, significantly decreased the cell surface amounts of both DR4 and DR5 in both cell lines; however, cell surface levels of DR4 were reduced much more substantially than cell surface DR5 by these inhibitors in both cell lines, as evidenced by comparing their mean fluorescence intensities (MFIs). Hence it is clear that MEK inhibition effectively decreases the levels of cell surface or functional DR4.

Pre-treatment of Cancer Cells with a MEK Inhibitor Impairs Cancer Cell Response to TRAIL-induced Apoptosis—Given that MEK inhibition decreases the amounts of cell surface DR5 and particularly DR4, we speculated that MEK inhibition might impair the ability of cancer cells to undergo TRAIL-induced apoptosis. Hence we pre-treated TRAIL-sensitive cancer cell lines with MEK162 for 18 h followed by exposing them to TRAIL for an additional 4 or 5 h. As shown in Fig. 3, we detected much lower amounts of DNA fragments (Fig. 3A) and cleaved caspase-8, caspase-3, and PARP (Fig. 3B) induced by TRAIL in cells pre-treated with MEK162 than in those without the pre-treatment. In agreement, significantly more cells survived in cells pre-treated with MEK162 than in matched control cells

when exposed to TRAIL (Fig. 3C). These results clearly demonstrate that pre-inhibition of MEK with a MEK inhibitor indeed impairs the ability of cancer cells to undergo TRAIL-induced apoptosis or protects cancer cells from being killed by TRAIL.

MEK Inhibitors Do Not Affect DR4 Protein Stability, but Reduce Its mRNA Levels—To begin to understand the mechanism by which MEK inhibition decreases DR4 levels, we examined the effects of MEK162 on DR4 and DR5 protein stabilities in H460 and HCT116 cells exposed to DMSO or MEK162 with a cycloheximide chase assay. We found that the degradation rates of both DR4 and DR5 in MEK162-treated cells and in DMSO-treated cells were comparable (Figs. 4, A and B), indicating that MEK162 does not alter the stabilities of both DR4 and DR5. Rather, we observed that MEK162 decreased DR4 and DR5 mRNA levels in both H460 and HCT116 cell lines (Fig. 4C), suggesting a possible transcriptional event or regulation.

MEK Inhibition with Genetic Gene Knockdown Decreases DR4 Expression and Protects Cancer Cells from TRAIL-induced Apoptosis—To further demonstrate the critical regulation of MEK/ERK signaling on DR4 expression, we knocked down MEK1, MEK2, or both and then examined their impact on DR4 expression and TRAIL-induced apoptosis. As presented in Fig. 5A, knockdown of MEK1, MEK2, or both substantially decreased the levels of DR4 in both H1299 and HCT116 cell lines. DR5 levels, particularly in H1299 cells, were also reduced upon MEK knockdown. We detected much less amounts of cleaved caspase-8, caspase-3, and PARP proteins (Fig. 5B) and DNA fragments (Fig. 5C) induced by TRAIL in cells transfected with both MEK1 and MEK siRNAs than in control siRNA-transfected cells. These data further validate our finding that inhibition of MEK decreases DR4 expression and protects cancer cells from TRAIL-induced apoptosis.

Activation of MEK/ERK Signaling Increases DR4 Levels—Suppression of DR4 expression by MEK inhibition demonstrated above suggests that MEK/ERK signaling may positively regulate DR4 expression. To confirm this regulation, we enforced activation of MEK/ERK signaling through transfection of B-RAF (V600E), constitutively activated MEK1 (MEK1-

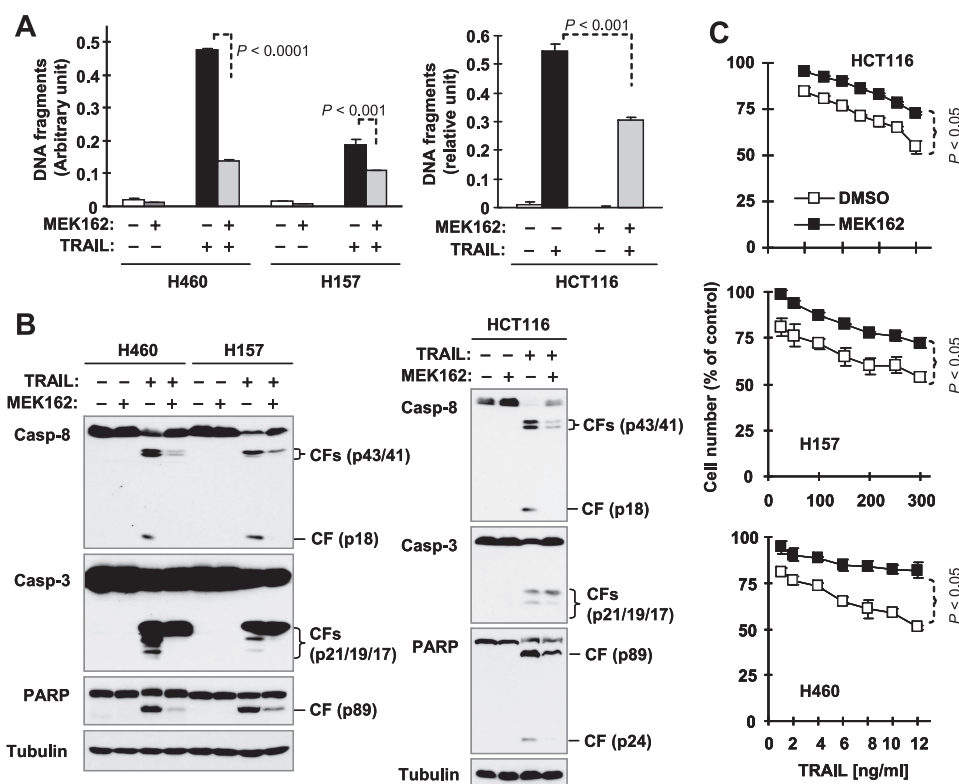


FIGURE 3. MEK162 attenuates the ability of TRAIL to induce apoptosis (A and B) and decrease cell viability (C) in TRAIL-sensitive cancer cells. A and B, the indicated cell lines were treated with DMSO or 1 μ M MEK162 for 18 h. After washing 3 times with PBS, the cells were then exposed to 10 (H460 and HCT116) or 200 ng/ml of TRAIL (H157) for an additional 4 (H460 and HCT116) or 5 h (H157). Whole cell protein lysates prepared from these treatments were finally used to measure histone-associated DNA fragments with a cell death ELISA kit (A) and to detect proteins of interest with Western blotting analysis (B). Columns represent mean \pm S.D. of triplicate determinations. CF, cleaved fragment. C, the indicated cell lines were pre-exposed to DMSO or 1 μ M MEK162 for 14 (H460) or 18 h (HCT116 and H157). After removing the medium, the cells were washed 3 times with PBS and then exposed to different concentrations of TRAIL as indicated for another 5 (H460) or 8 h (HCT116 and H157). Cell numbers were estimated with the sulforhodamine B assay and data are presented as mean \pm S.D. of triplicate determinations.

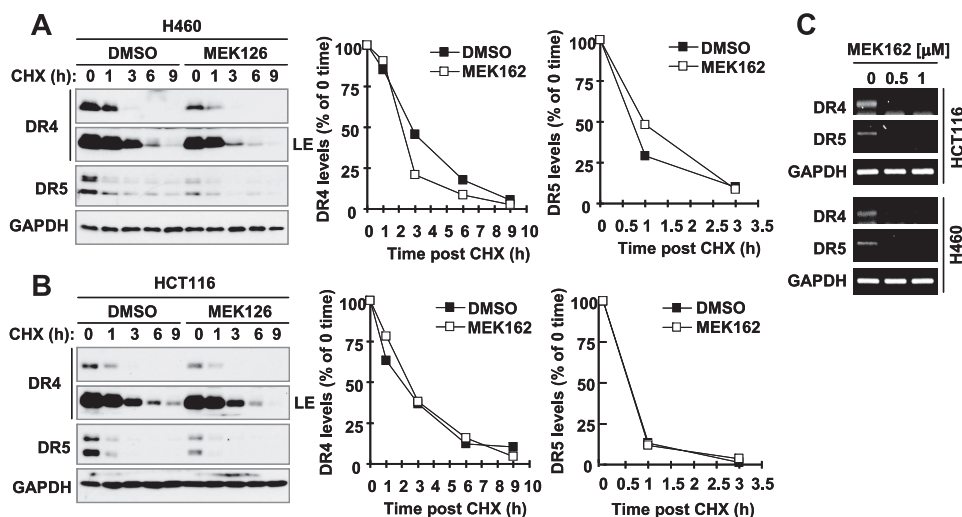


FIGURE 4. MEK162 does not alter protein stability of DR4 and DR5 (A and B), but decreases mRNA levels of both DR4 and DR5 (C) in cancer cells. A and B, both H460 (A) and HCT116 (B) cell lines were exposed to 1 μ M MEK162 for 8 h followed by treatment with 10 μ g/ml of cycloheximide (CHX). At the indicated times post-CHX, the cells were harvested for preparation of whole cell protein lysates and subsequent Western blotting analysis. Protein levels were quantified with NIH Image J software and normalized to GAPDH. The results were plotted as relative DR4 or DR5 levels compared with those at time 0 of CHX treatment (right panels). LE, longer exposure. C, the indicated cell lines were treated with the given concentrations of MEK162 for 8 h and then harvested for preparation of total cellular RNA and subsequent RT-PCR.

CA) or MEK2 (MEK2-CA), and then examined their impact on DR4 expression. Transfection of B-RAF (V600E) into H1299 cells increased p-ERK1/2 levels accompanied with elevation of both DR4 and DR5 (Fig. 6A). Similarly, enforced expression of

MEK1-CA or MEK2-CA in H1299 cells also increased the levels of not only p-ERK1/2, but also DR4 and DR5 (Fig. 6B). Under these conditions, DR4 mRNA levels were also significantly increased (Fig. 6C). The presence of MEK162 decreased basal

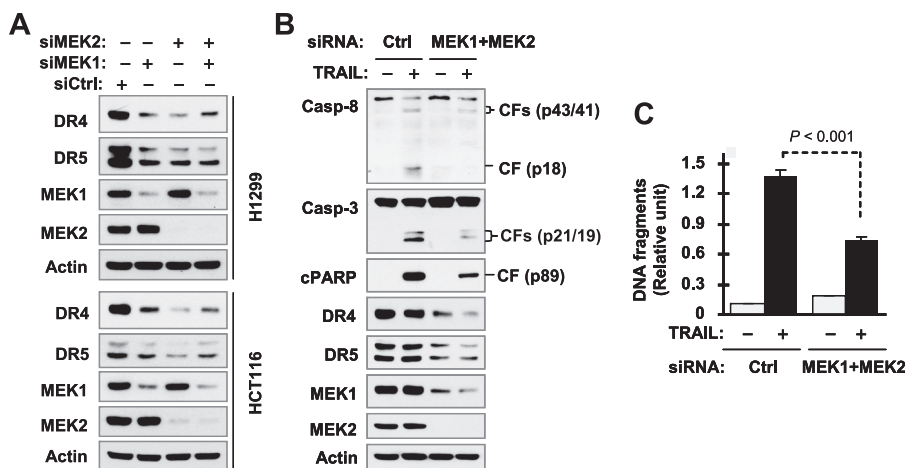


FIGURE 5. Inhibition of MEK by knockdown of MEK1, MEK2, or both decreases the levels of DR4 and DR5 (A) and protects cancer cells from undergoing TRAIL-induced apoptosis (B and C). A, the given siRNAs were transfected into the indicated cancer cell lines for 48 h. B and C, HCT116 cells were transfected with the indicated siRNAs for 30 h followed with TRAIL (7.5 ng/ml) treatment for another 3 h. After these procedures, the cells were harvested for Western blotting analysis to detect the interested proteins as indicated (A and B) or for ELISA to detect DNA fragments (C). Each column represents mean \pm S.D. of triplicate determinations.

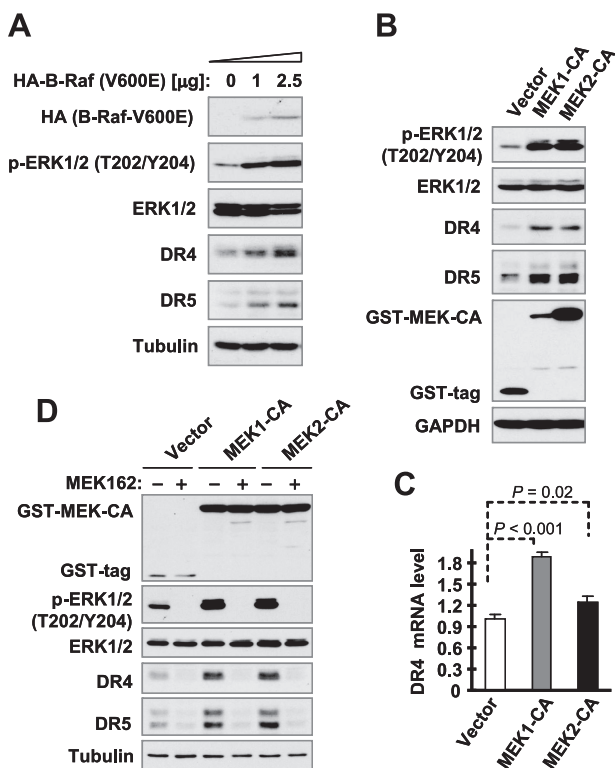


FIGURE 6. Enforced activation of MEK/ERK signaling by expressing ectopic mutant *B-RAF* (A) or constitutively activated *MEK1* (*MEK1-CA*) or *MEK2* (*MEK2-CA*) (B and C) increases *DR4* expression in a MEK-dependent fashion (D). A and B, H1299 cells were transfected with the indicated plasmids for 48 h. D, H1299 cells were transfected with the indicated plasmids and after 43 h were then exposed to DMSO or 1.5 μ M MEK162 for an additional 7 h. After the aforementioned transfections or treatments, the cells were harvested for preparation of whole cell protein lysates and subsequent Western blotting analysis. C, H1299 cells were transfected with the given plasmids for 30 h. Total RNA were then prepared from these cells and used for detection of *DR4* mRNA with quantitative RT-PCR. Each column is mean \pm S.D. of triplicate determinations.

levels of p-ERK1/2, DR4, and DR5, and inhibited elevation of these proteins induced by MEK1-CA or MEK2-CA (Fig. 6D), indicating a MEK-dependent activation of ERK1/2 and elevation of DR4 and DR5. Together we conclude that activation of

MEK/ERK signaling indeed positively regulates *DR4* and *DR5* expression.

MEK/ERK Signaling Activation Positively Regulates DR4 Expression through Enhancing AP-1-mediated Transcription—To further elucidate the mechanism by which MEK/ERK signaling positively regulates *DR4* expression, we then determined whether MEK activation enhances *DR4* promoter activity. Co-transfection of *MEK1-CA* with the luciferase reporter vector carrying the 5'-flanking region of the *DR4* gene (-1121) into HEK293T cells significantly increased luciferase activity. This enhanced effect could be abolished by the presence of MEK162 or AZD6244 (Fig. 7A), indicating MEK activation-dependent effects. Hence it is clear that MEK/ERK activation enhances *DR4* gene transactivation. To further narrow down the essential region that is responsible for driving MEK/ERK-dependent *DR4* gene expression, we examined the effects of MEK1-CA on the activation of *DR4* reporter vectors harboring different lengths of *DR4* 5'-flanking regions. MEK1-CA substantially increased luciferase activity (\sim 150%) in cells transfected with pGL3-*DR4* (-1773/+63)-luc, pGL3-*DR4* (-1121/+63)-luc, pGL3-*DR4* (-586/+63)-luc, but limited (\sim 50%) in cells transfected with pGL3-*DR4* (-208/+63)-luc although the increase was still statistically significant (Fig. 7B). These results suggest that the region between -586 and -208 in the *DR4* 5'-flanking region is essential for mediating MEK-dependent *DR4* gene transactivation. In other words, the response elements in this region and proteins that bind to these elements are responsible for MEK-dependent *DR4* gene transactivation. In this region, there is a functional AP-1 binding site (-351/-344) and another putative AP1 binding site (-487/-481) as demonstrated in our previous study (14). Considering that MEK/ERK signaling activates AP-1 (29, 30), we determined whether the AP-1 sites in this region are required for MEK to transactivate the *DR4* gene. Mutation of the AP-1 site at -350/-344, but not at -487/-481, abolished the ability of MEK1-CA to increase *DR4* promoter activity (Fig. 7C), indicating that the AP-1 site at -350/-344 is responsible for MEK-dependent *DR4* gene transactivation.

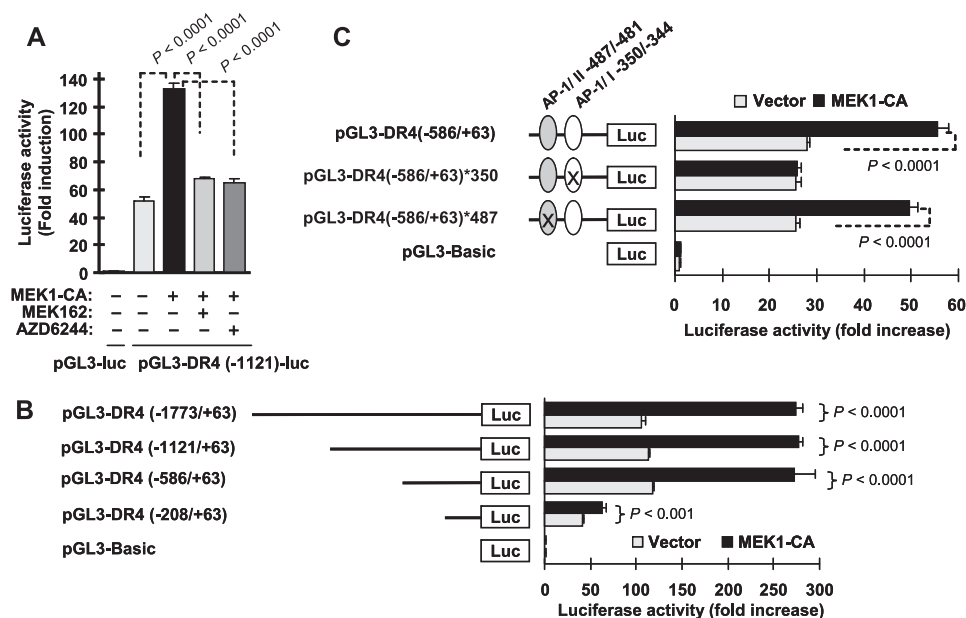


FIGURE 7. MEK activation induces MEK- and AP-1-dependent DR4 gene transactivation. A, 293T cells were co-transfected with the indicated DR4 reporter plasmids plus MEK1-CA and after 18 h were then exposed to DMSO or 2 μ M MEK162 or AZD6244 for an additional 10 h. B and C, 293T cells were co-transfected with the given DR4 reporter plasmids plus MEK1-CA. Approximately 30 h after these transfections, the cells were lysed for luciferase activity assay. The data are mean \pm S.D. of triplicate determinations.

It is known that AP-1 is primarily composed of c-Jun and Fos proteins (29). To further demonstrate the critical role of AP-1 in mediating MEK activation-mediated DR4 gene transactivation and expression, we then examined the impact of c-Jun knockdown on MEK-dependent DR4 transactivation and expression. As presented in Fig. 8A, knockdown of c-Jun with two different small interfering RNAs (siRNAs) abrogated the ability of MEK1-CA to transactivate the DR4 gene. Moreover, knockdown of c-Jun blocked the increase in DR4 induced by enforced expression of MEK1-CA (Fig. 8B). We noted that knockdown of c-Jun did not affect ERK activation induced by MEK1-CA (Fig. 8B), indicating that AP-1 activation is a downstream event of ERK activation. We also observed that enforced MEK1-CA expression substantially increased the levels of both p-c-Jun and c-Jun, suggesting activation of AP-1 by MEK/ERK signaling in the tested cell system.

Inhibition of MEK with MEK Inhibitors Decreases DR4 Levels Accompanied with Suppression of c-Jun Expression—To demonstrate that MEK inhibitors decrease DR4 expression through suppression of a MEK/ERK/AP-1-dependent mechanism, we further examined the effects of MEK inhibitors on c-Jun expression. We found that the 3 tested MEK inhibitors, MEK162, AZD6244, and PD0325901, effectively decreased the levels of p-c-Jun and c-Jun (Fig. 8C). This data suggests that MEK inhibitors indeed inhibit c-Jun/AP-1. Hence we assume that MEK inhibition with MEK inhibitors decreases DR4 levels through suppression of AP-1-dependent DR4 expression.

Discussion

Our previous studies have demonstrated that DR5 expression is positively regulated by MEK/ERK signaling through increasing CHOP/Elk-dependent gene transcription (25, 26). Accordingly, inhibition of this signaling pathway down-regulates DR5 expression and attenuates DR5 activation-induced

apoptosis including TRAIL-induced apoptosis (27). The current study further demonstrates that DR4, the sibling of DR5, is also a gene whose expression is positively regulated by MEK/ERK signaling and can be inhibited by blocking this signaling pathway, e.g. with MEK inhibitors as demonstrated (Figs. 1, 2, and 5–8).

Similar to DR5 regulation, DR4 regulation by MEK/ERK signaling also occurs at the transcriptional level, but through a different mechanism. It is known that ERK can directly activate AP-1 and mediates its gene transactivation through direct phosphorylation of c-Jun (31, 32) and enhancement of c-Jun expression and stability (33). In this study, we found that the presence of an AP-1 binding site in the DR4 5'-flanking region is essential for DR4 transactivation induced by MEK activation (Fig. 7). Consistently, inhibition of AP-1 by knockdown of c-Jun not only blocked DR4 gene transactivation, but also prevented DR4 up-regulation induced by MEK activation (Fig. 8). These results together indicate an essential role of AP-1 in mediating DR4 expression induced by activation of MEK/ERK signaling. Therefore the MEK/ERK/AP-1 signaling pathway represents a previously undiscovered mechanism for the positive regulation of DR4 expression. This finding is further supported by our demonstration that MEK activation elevated the levels of p-c-Jun and c-Jun while increasing DR4 expression (Fig. 8B), whereas MEK inhibition decreased DR4 levels accompanied with suppression of c-Jun phosphorylation and expression (Fig. 8C).

Endogenous TRAIL and DR5 or DR4 interaction leading to the induction of apoptosis is recognized as a critical mechanism of immune surveillance against cancer cells (34, 35). Hence defective death receptor signaling may favor immune escape and survival of cancer cells. Our current finding that MEK inhibition causes DR4 down-regulation in cancer cells together

MEK/ERK Signaling and DR4 Expression

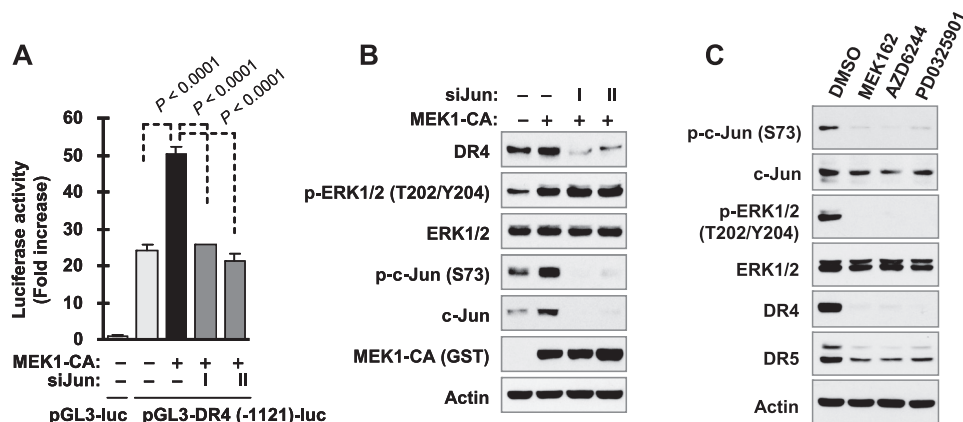


FIGURE 8. MEK activation increases c-Jun-dependent DR4 gene transactivation (A) and expression (B) with elevated levels of p-c-Jun and c-Jun, whereas MEK inhibition decreases DR4 expression accompanied with decreased levels of p-c-Jun and c-Jun (C). A, 293T cells were co-transfected with the indicated DR4 reporter plasmids, MEK1-CA, and different c-Jun siRNAs, and were harvested after 30 h for luciferase assay. The data are mean \pm S.D. of triplicate determinations. B, H1299 cells were transfected with MEK1-CA and after 20 h were transfected with the indicated c-Jun siRNAs and incubated for an additional 36 h. The cells were then harvested for preparation of whole cell protein lysates and subsequent Western blotting analysis. C, HCT116 cells were treated with DMSO or 1 μ M MEK inhibitors as indicated for 12 h before the cells were harvested for preparation of whole cell protein lysates and subsequent Western blotting analysis.

with our previous demonstration of B-RAF or MEK inhibition-induced suppression of DR5 expression in cancer cells (27) and the impairment of cancer cell response to TRAIL-induced apoptosis by MEK inhibition (Figs. 3 and 5) (27) may suggest that the long-term treatment of cancers with a MEK inhibitor may compromise the immune surveillance of cancer cells or encourage their escape from immune surveillance. As a consequence, this may lead to increased metastasis or the appearance of secondary malignancies. Immunotherapy represents an attractive and effective strategy against cancers such as melanoma and is believed to involve killing of cancer cells by the induction of apoptosis. One of the primary underlying mechanisms involves death ligand-induced apoptotic signaling, mainly by TRAIL from T cells, monocytes, and dendritic cells (37, 38). Hence our findings in this study also raise important concerns for a potential negative impact of MEK-targeted therapy on the clinical efficacy of salvage treatment with immune-based cancer therapy. Thus, further preclinical and clinical studies to clarify and address these concerns should be conducted in the future.

Materials and Methods

Reagents—MEK162 (binimetinib) was provided by Novartis Pharmaceuticals Corporation (East Hanover, NJ). AZD6244 (selumetinib, ARRY-142886) and PD0325901 were purchased from Selleckchem (Houston, TX). Human recombinant TRAIL was purchased from PeproTech, Inc. (Rocky Hill, NJ). Mouse monoclonal anti-DR4 antibody (B-N28) was purchased from Cell Sciences (Canton, MA). p-c-Jun (S73), c-Jun, and HA tag antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). GST tag, MEK1 and MEK2 antibodies were purchased from Sigma. Other antibodies were the same as described previously (25).

Cell Lines—Human lung cancer cell lines used in this study were described previously (28). HCT116 was purchased from American Type Culture Collection (ATCC, Manassas, VA). HEK293T cells were provided by K. Ye (Emory University, Atlanta, GA). Except for H157, A549, H460, and H1299, other cell lines were not authenticated. These cell lines were grown in

RPMI 1640 medium supplemented with 5% fetal bovine serum at 37 °C in a humidified atmosphere consisting of 5% CO₂.

Detection of Apoptosis—Apoptosis was evaluated with a Cell Death Detection ELISA^{PLUS} kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Cleavage of caspases and PARP was detected by Western blotting analysis as additional indications of apoptosis.

Cell Viability Assay—The given cells were seeded in 96-well cell culture plates, exposed to different treatments and then subjected to estimation of cell numbers by the sulforhodamine B assay as previously described (39).

Western Blotting Analysis—Whole cell protein lysates were prepared and analyzed by Western blotting analysis as described previously (25).

Detection of Cell Surface DR4 and DR5—Cell surface DR4 or DR5 expression was detected with flow cytometry as described previously (40). The MFI that represents antigenic density on a per cell basis was used to assess cell surface DR4 and DR5 levels. Phycoerythrin (PE)-conjugated mouse anti-human DR5 (DJR2-4), anti-human DR4 (DJR1) monoclonal antibodies, and PE mouse IgG1 isotype control (MOPC-21/P3) were purchased from eBioscience (San Diego, CA).

Detection of DR4 and DR5 mRNA Expression—DR4 and DR5 mRNAs were detected with RT-PCR (19, 27) or quantitative RT-PCR as described previously (19).

Reporter Plasmids and Luciferase Activity Assay—All DR4 reporter constructs used in this study were described previously (14). Plasmid transfection and luciferase assays were the same as described previously (14).

Expression Constructs and Transfection—B-RAF (V600E) expression construct was the same as described previously (26). Expression plasmids harboring constitutively activated MEK1 (MEK1-CA) or MEK2 (MEK2-CA) were made using a site-directed mutagenesis strategy as follows: primers (MEK1/MEK2-CA forward, 5'-ctcatcgacgacatggccaacgactctgtgggc-3' and MEK1/MEK2-CA reverse, 5'-gccacgaagctgtggccatgctgctgatgag-3') designed to mutate MEK1 in plasmid pDONR

223 MEK1 (number 23406; Addgene, Cambridge, MA) at Ser²¹⁸ and Ser²²² positions into Asp²¹⁸ and Asp²²², and MEK2 in plasmid pDONR 223 MEK2 (number 23555; Addgene) at the Ser²²² and Ser²²⁶ positions into Asp²²² and Asp²²⁶ were purchased from Integrated DNA Technologies (Coralville, IA). Site-directed mutagenesis was performed using a QuikChange II XL Site-directed Mutagenesis kit and specifications (Agilent, Santa Clara, CA). Mutated pDONR plasmids were confirmed by sequencing and cloned into destination vector pDEST27 (number 11812-013; ThermoFisher Scientific, Waltham, MA) using Gateway LR Clonase Enzyme Mix (number 11791-020, ThermoFisher Scientific). Cell transfection with the given plasmids was conducted using PolyJet DNA transfection reagent (SigmaGen Laboratories, Rockville, MD) or Lipofectamine 2000 (ThermoFisher Scientific/Invitrogen, Grand Island, NY) according to the manufacturer's protocol.

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) was described previously (36). c-Jun (numbers 6205 and 6204), MEK1 (number 6426) and MEK2 (number 6431) siRNAs were purchased from Cell Signaling Technology, Inc. Gene silencing effects were evaluated by Western blotting analysis as described above.

Statistical Analysis—The statistical significance of differences between two experimental groups was determined with two-sided unpaired Student's *t* test using GraphPad InStat 3 software. Results were considered to be statistically significant at $p < 0.05$. To maintain the overall significance level at 0.05 when there are multiple tests, the *p* value for individual tests was adjusted as $0.05/n$ for *n* multiple tests using the Bonferro's correction.

Author Contributions—W. Y. and Y. T. O. designed and conducted most the experiments. J. D. helped with assaying cell surface receptors. L. D. generated some data used in Fig. 1. P. Y. prepared reagents and provided technical assistance. H. H. and W. Z. constructed constitutively activated MEK1 and MEK2. S. Y. S. conceived and coordinated the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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