Synthetic lethal targeting of *MYC* by activation of the DR5 death receptor pathway

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Summary

The genetic concept of synthetic lethality provides a framework for identifying genotype-selective anticancer agents. In this approach, changes in cellular physiology that arise as a consequence of oncogene activation or tumor suppressor gene loss, rather than oncoproteins themselves, are targeted to achieve tumor selectivity. Here we show that agonists of the TRAIL death receptor DR5 potently induce apoptosis in human cells overexpressing the *MYC* oncogene, both in vitro and as tumor xenografts in vivo. MYC sensitizes cells to DR5 in a p53-independent manner by upregulating DR5 cell surface levels and stimulating autocatalytic processing of procaspase-8. These results identify a novel mechanism by which MYC sensitizes cells to apoptosis and validate DR5 agonists as potential MYC-selective cancer therapeutics.

Introduction

The *MYC* proto-oncogene encodes a basic helix loop helix zipper (bHLHZ) domain-containing transcription factor that plays a critical role in cellular proliferation (for review, see Grandori et al., 2000). Induced rapidly upon entry into the cell cycle, MYC expression is sufficient to drive quiescent cells into proliferation. Although *MYC* was originally characterized as a human oncogene based on translocation of its genomic locus in Burkitt's lymphoma, subsequent evidence indicates that aberrant MYC expression occurs by a wide range of mechanisms in a variety of different tumor types (Nesbit et al., 1999; Oster et al., 2002). Indeed, MYC has been proposed to be deregulated in a large percentage of human cancers, and its overexpression frequently correlates with aggressive, poorly differentiated tumors and poor prognosis. Paradoxically, in addition to promoting proliferation, MYC is also capable of sensitizing cells to apoptotic cell death under certain conditions (Evan et al., 1992; Pelengaris et al., 2002). While the precise mechanism by which this occurs remains unclear, this ability is believed to result from the existence of cellular tumor suppressor mechanisms that are "hard-wired" to restrain the growth of cells that have acquired oncogenic mutations.

Because MYC is deregulated in many human tumors, its ability to sensitize cells to apoptosis could potentially provide a therapeutic window that would allow MYC-overexpressing tumor cells to be killed preferentially over normal cells. Agents that could exploit this hypersensitivity would achieve tumor specificity by a mechanism distinct from directly targeting oncoprotein function, as molecularly targeted therapeutics such as Gleevec do (Capdeville et al., 2002; Druker, 2002). Instead, they would target an oncogene-dependent sensitivity. Such a strategy represents a pharmacogenetic approach analogous to the concept of genetic synthetic lethality (Hartwell et al., 1997), a term that refers to nonviability resulting from the combined presence of two mutations or alleles, neither of which by itself is sufficient to induce death. By analogy, the pharmacogenetic version of this concept would refer to a drug that does not affect wild-type cells, but induces lethality in cells carrying a specific genetic alteration, such as a *MYC* translocation or amplification (Reddy and Kaelin, 2002). Currently, a handful of attempts to identify such genotype-specific agents have been reported, targeting cells that carry alterations in cancer-associated pathways such as receptor-tyrosine kinase/RAS signaling pathways (Fantin et al., 2002; Torrance et al., 2001) and/or in the p53 or retinoblastoma (pRB) tumor suppressor pathways (Dolma et al., 2003).

The cytokine TRAIL (Tumor Necrosis Factor Related Apoptosis Inducing Ligand, also known as APO2L) has received much attention as a potential cancer therapeutic because it is capable of inducing apoptosis in a wide variety of tumor cells while sparing most normal cell types (reviewed in Ashkenazi,

SIGNIFICANCE

Using a series of human cells carrying defined oncogenic genetic alterations, we show that activation of the TRAIL receptor, DR5, induces apoptotic cell death preferentially in cells overexpressing MYC, both in vitro and in vivo. We thereby demonstrate that MYC's well-established ability to sensitize cells to apoptosis provides a therapeutic window that can be exploited to target a pharmacological agent's action specifically toward MYC-overexpressing tumor cells in vivo. In addition, our results provide insight into the long unanswered question of why many tumor cells are sensitive to DR5-mediated apoptotic signaling while most normal cells are resistant. Since MYC overexpression occurs frequently in human cancer, DR5 agonists, which are currently undergoing clinical development, may be effective against a wide variety of tumor types.

2002; Ozoren and El-Deiry, 2003). While the molecular basis for this selectivity is unknown, TRAIL appears to play a role in immune surveillance against cancer (Smyth et al., 2003), suggesting that one of its physiological functions is to recognize and eradicate tumor cells. A homotrimeric type II transmembrane protein and member of the tumor necrosis factor (TNF) family of "death ligands," TRAIL induces apoptosis by engaging receptors on the cell surface and activating an intracellular cascade of cell death-inducing proteases known as caspases through both mitochondria-dependent and -independent pathways (Ashkenazi, 2002).

Five TRAIL receptors have been identified to date. Of these, the Death Receptors 4 (DR4, also known as TRAIL-R1, APO2) and 5 (DR5, also known as TRAIL-R2, TRICK2, KILLER) contain cytoplasmic death domains and can facilitate activation of the initiator caspase, caspase-8, while the others, the so-called decoy receptors DcR1, DcR2, and OPG, lack intact death domains (Ashkenazi, 2002; Ozoren and El-Deiry, 2003). Functionally inactive, these decoy receptors can compete with DR4 and DR5 for binding to TRAIL and may therefore protect some cell types from exposure to the TRAIL ligand.

Here we show that agonists of DR5 can act as pharmacological agents that are synthetically lethal with MYC overexpression, potently inducing apoptosis in human cells overexpressing the *MYC* oncogene, both in vitro and in tumor xenografts in vivo. These results identify *MYC* as a critical determinant of TRAIL's DR5-mediated tumor specificity and suggest that DR5 agonists may be effective as *MYC*-selective cancer therapeutics.

Results

MYC sensitizes normal human fibroblasts to TRAIL-induced apoptosis

MYC's ability to sensitize cells to apoptotic stimuli has been well documented, particularly in rodent fibroblasts (Pelengaris et al., 2002). However, some strains of normal human fibroblasts overexpressing MYC have been reported to resist apoptosis under serum starvation conditions that are sufficient to induce cell death in rodent cells (Vafa et al., 2002). To systematically examine MYC's ability to sensitize nontransformed human cells to apoptosis, we subjected BJ primary human foreskin fibroblasts, as well as derivative cells, BJ-MYC, carrying a retrovirally introduced *MYC* oncogene, to a variety of apoptotic stimuli. Of the treatments tested, which included serum starvation, DNA-damaging chemotherapeutic drugs, ionizing radiation, and activators of the TNF, FAS, and TRAIL death receptor signaling pathways, only TRAIL was able to induce cell death selectively in BJ-MYC, causing $>$90% decrease in viability, while having no effect on BJ cells infected with vector alone (Figure 1A). DNA-damaging agents and serum starvation also resulted in decreases in viable cell counts when compared to untreated cells. However, these decreases were primarily due to cell cycle arrest rather than cell death, as determined by microscopic observation of nuclear morphology and Hoechst 33342 staining (data not shown), and were only very modestly enhanced by MYC overexpression (Figure 1A).

TRAIL acts through the DR5 receptor to induce apoptosis in MYC-overexpressing cells

To determine through which receptor(s) TRAIL acts to induce cell death in BJ-MYC cells, we tested the ability of DR4- and DR5-specific antagonistic monoclonal antibodies (mAbs) (Leverkus et al., 2003; Sprick et al., 2002) to block TRAIL-mediated killing (Figure 1B). The anti-DR4 mAb, HS101, failed to protect BJ-MYC from TRAIL-induced apoptosis. In contrast, the anti-DR5 mAb, HS201, strongly protected cells. Addition of both mAbs did not result in additional protection over that provided by anti-DR5 alone, suggesting that TRAIL acts primarily through the DR5 receptor, but not DR4, to induce apoptosis in BJ-MYC cells.

Additional evidence in support of this conclusion was obtained using independently isolated anti-DR4 and -DR5 mAbs (named DR4-A and DR5-A, respectively). In contrast to HS101 and HS201, these mAbs act as receptor agonists and are each capable of inducing apoptosis with similar efficiencies in TRAIL-sensitive HCT116 colon carcinoma cells (D.A.K. and M.N., unpublished data). Their specificity for their cognate receptors allows the role of each receptor to be assessed independently of the other or of any potential protective effects by decoy receptors (D.A.K. and M.N., unpublished data; Griffith et al., 1999; Ichikawa et al., 2001). Similar to their response to TRAIL, BJ-MYC cells were dramatically sensitized to DR5-A, whereas BJ cells were largely resistant (Figures 1C and 1D). However, both control and MYC-expressing cells were completely resistant to DR4-A, confirming that MYC sensitizes BJ cells to apoptotic signaling through the DR5 receptor, but not through DR4. The sensitivity of BJ-MYC to DR5-A was unlikely to be due to increased cell proliferation caused by MYC, because co-expression of the cyclin-dependent kinase inhibitor $p27^{KIP1}$ decreased cell cycle progression, yet did not abolish or reduce sensitivity to DR5-A (Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/5/5/501/DC1).

To determine whether the pharmacogenetic interaction between DR5 agonists and MYC deregulation is a general phenomenon in human cells, we tested three additional strains of primary or genetically defined cells: two primary human fetal lung fibroblasts, IMR90 and WI38, and a nontransformed human embryonic kidney epithelial cell line, HA1E, immortalized by expression of the telomerase catalytic subunit TERT and the SV40 large T antigen (SV40 LT). Each of these lines behaved similarly to BJ cells, being resistant to TRAIL ligand, DR5-A, and DR4-A in the absence of MYC overexpression (Figure 2). However, their MYC-expressing counterparts were all highly sensitive to TRAIL and DR5-A, but not DR4-A, suggesting that MYC-induced sensitization to DR5 signaling is a general phenomenon occurring in human cells of both mesenchymal and epithelial origin.

MYC stimulates caspase-8 autocatalytic processing

Both DR4 and DR5 are thought to transmit apoptotic signals in a similar manner (see Figure 3A). In response to ligand binding, a death-inducing signaling complex (DISC) composed of trimeric ligand, receptor, and the adaptor protein FADD rapidly recruits the initiator caspase procaspase-8 (Peter, 2000). Proximity within the DISC facilitates dimerization of procaspase-8, resulting in activation and proteolytic self-processing (Boatright et al., 2003). Active caspase-8 can, in cell types termed type I cells, directly cleave and activate downstream effector caspases such as caspase-3 that execute the apoptotic death program (Fulda et al., 2002; Scaffidi et al., 1998). Alternatively, in type II cells, active caspase-8 is insufficient to directly activate effector caspases, but instead cleaves the pro-apoptotic Bcl-2 family

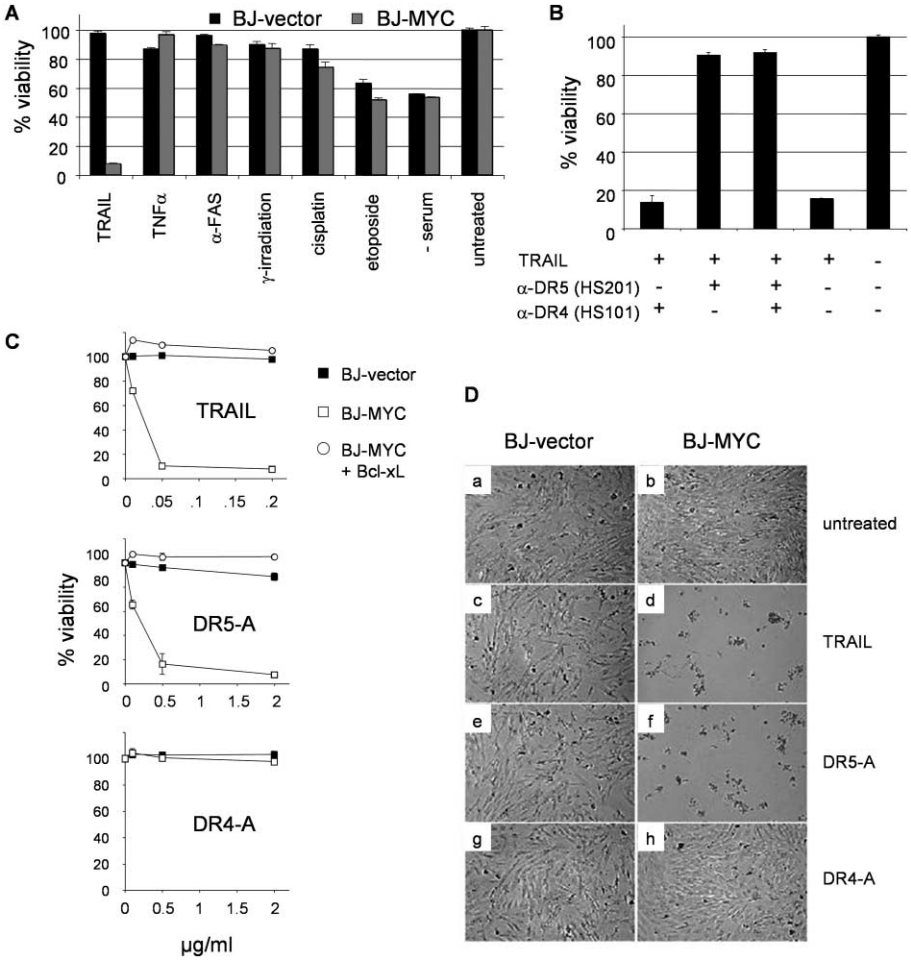

Figure 1. MYC sensitizes normal human fibroblasts to TRAIL-induced cell death via the DR5 receptor

A: BJ human foreskin fibroblasts carrying either a stably integrated retroviral MYC expression construct (BJ-MYC) or empty vector (BJ-vector) were treated overnight with various apoptosis-inducing stimuli and assayed for cell viability using CellTiter-Glo (Promega).

B: Effect of anti-DR4 and anti-DR5 blocking antibodies HS101 and HS201 on TRAIL-induced apoptosis. BJ-MYC cells were pretreated for 1 hr with 10 μg/ml blocking antibody and then stimulated overnight with 50 ng/ml TRAIL.

C: Effect of TRAIL and agonistic anti-DR4 and anti-DR5 antibodies (DR4-A and DR5-A, respectively) on BJ, BJ-MYC, and BJ-MYC + pBABE-Bcl-xL. In all experiments, error bars indicate standard deviations (SD) of triplicate measurements. The absence of visible error bars indicates that the SD was smaller than the representative symbol.

D: Photomicrographs of BJ and BJ-MYC fibroblasts treated with TRAIL (200 ng/ml), DR5-A (2 μg/ml), or DR4-A (2 μg/ml) for 16 hr.

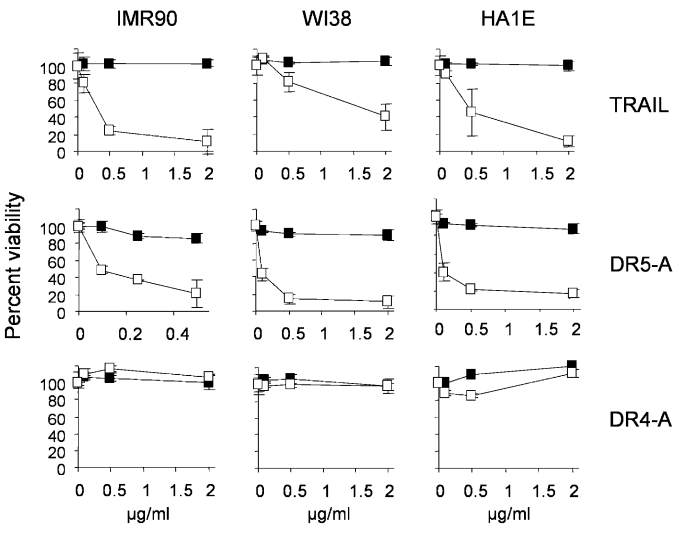

Figure 2. MYC sensitizes multiple human cell types to TRAIL-induced cell death via DR5

Human fetal lung fibroblasts (IMR90 and WI38) and immortalized embryonic kidney cells (HA1E), all carrying either MYC-expressing (open square) or empty vector (closed square) proviruses, were treated with TRAIL, DR5-A, or DR4-A for 16 hr at the indicated concentrations.

member Bid to its active form, tBid, which engages the mitochondrial, or intrinsic, cell death pathway (LeBlanc et al., 2002; Scaffidi et al., 1998). This results in the release of pro-apoptotic factors such as cytochrome *c* and SMAC/DIABLO from the mitochondria and subsequent activation of caspase-9 and downstream effector caspases such as caspase-3.

To explore the mechanistic basis for the synthetic lethal interaction between DR5-A and *MYC*, we examined the apoptotic signaling pathway in normal human fibroblasts in the presence or absence of ectopically expressed MYC. Upon DR5-A stimulation, vector-transduced IMR90 and WI38 fibroblasts showed no processing of any of the caspases examined (Figure 3B). In contrast, MYC-expressing cells rapidly initiated the caspase proteolytic cascade, resulting in processing of caspase-8, -9, and -3 and Bid within 4 hr. As Bid and caspase-9 act specifically in the mitochondrial pathway, the observed cleavage of these proteins suggests that apoptosis in these cells involved the engagement of the mitochondrial pathway. However, since cells lacking ectopic MYC expression did not exhibit autocatalytic processing of the initiator caspase, caspase-8, or cleavage of its substrate Bid, these results suggest that MYC acts by relieving a barrier to apoptosis in normal cells at or upstream of caspase-8 activation.

In type II cells, activation of downstream effector caspases by the mitochondrial pathway can result in further cleavage and activation of caspase-8 (Scaffidi et al., 1998), potentially creating

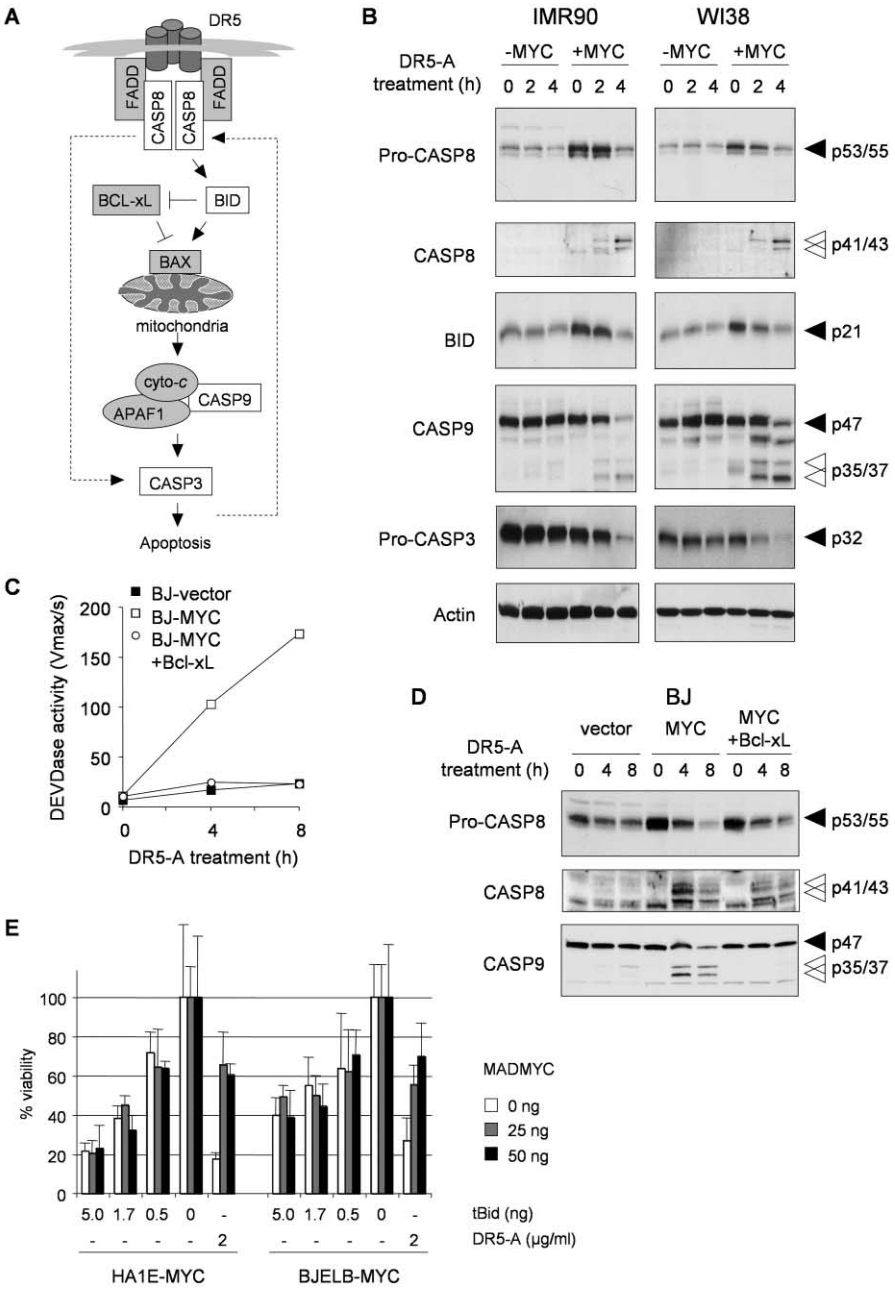

Figure 3. MYC stimulates autocatalytic processing of caspase-8

A: Schematic of the DR5 apoptotic signaling pathway. Dotted lines represent interactions that do not appear to play a major role in the cell types examined in this study, but which are present in some cell types. See text for details.

B: Immunoblots showing proteolytic processing of the indicated apoptotic signaling components in MYC-expressing and control fibroblasts treated with 0.5 μg/ml DR5-A for the indicated times. Filled arrows to the right indicate unprocessed forms, open arrows indicate active processed forms.

C: Effect of MYC and Bcl-xL on effector caspase (DEVDase) activity in BJ cells.

D: Caspase-8 processing induced by MYC and DR5-A is mitochondria independent. Immunoblots showing caspase-8 and -9 processing in BJ-MYC cells in which mitochondrial apoptotic function was blocked by Bcl-xL.

E: The dominant-negative *MYC* allele, *MADMYC*, blocks apoptosis induced by DR5-A, but not tBid. The indicated amounts of MADMYC were transiently transfected to repress MYC target gene expression. Apoptosis was induced by cotransfection of a tBid expression construct or by treatment with 2 μg/ml DR5-A antibody.

a positive feedback loop that amplifies the apoptotic signal (see Figure 3A). Thus, formally, the stimulation of caspase-8 processing by MYC, rather than being due to enhancement of autocatalytic activity at the DR5 DISC, could be due to enhancement of the mitochondrial pathway, as has been proposed to occur in rodent fibroblasts (Juin et al., 1999). To exclude this possibility, we retrovirally introduced into BJ-MYC a cDNA encoding the anti-apoptotic Bcl-2 family member Bcl-xL, which inhibits apoptosis by negatively regulating mitochondrial membrane permeability, thereby preventing the release of pro-apoptotic factors. Bcl-xL strongly inhibited effector caspase activity (Figure 3C) and cell death (Figure 1C) in BJ-MYC cells, demonstrating that apoptosis is mitochondria dependent and proceeds by a type II mechanism in these cells. The near complete suppression of cell death, accompanied by lack of detectable caspase-9 processing (Figure 3D), suggests that mitochondrial apoptotic signaling was almost totally abolished by Bcl-xL. However, under these conditions, and thus in the absence of any mitochondrial amplification loop, caspase-8 processing was still strongly stimulated by DR5-A in BJ-MYC cells (Figure 3D). These results therefore suggest that MYC acts at the level of caspase-8 activation within the DR5 DISC.

To obtain further evidence that enhancement of caspase-8 activity by MYC was independent of mitochondrial amplification, we inhibited MYC transcriptional activity using a dominant-negative version of MYC, MADMYC, comprised of the transcriptional repression domain of the MYC/MAX antagonist MAD fused to the DNA binding and dimerization domain of MYC

(Berns et al., 1997). Ectopic expression of MADMYC via transfection resulted in up to 80% decreased transcription from a cotransfected MYC-responsive luciferase reporter construct (data not shown) and strongly reduced sensitivity to DR5-A (Figure 3E). However, apoptosis induced by ectopic expression of the processed form of Bid, tBid, was completely insensitive to the inhibition of MYC transcriptional activity (Figure 3E). These results were observed using a MYC-expressing, immortalized derivative of BJ, BJELB-MYC, as well as the epithelial cell line HA1E-MYC, suggesting that in human cells, MYC acts upstream of Bid processing in the DR5 death receptor pathway.

MYC upregulates the DR5 receptor

The ability of MYC to sensitize BJ cells to activation of DR5 preferentially over the related receptors DR4 or FAS, which utilize similar signaling pathways, suggests that MYC activity might impinge upon the TRAIL signaling pathway at the level of the DR5 receptor itself. DR5 protein was readily detectable in normal human cells by immunoblotting but became upregulated approximately 2-fold upon MYC overexpression (Figure 4A and Supplemental Figure S2 at http://www.cancercell.org/cgi/content/full/5/5/501/DC1), an effect that could be reversed by retroviral MADMYC expression. The increase in total DR5 protein levels resulted in increased cell surface DR5 receptor levels, as determined by flow cytometry on intact cells (Figure 4B). DR4, FAS, and TNF receptor 1 (TNFR1) surface levels all remained unaffected by MYC, demonstrating that MYC's effect upon cell surface death receptor levels was specific to DR5.

To determine if MYC upregulates DR5 expression at the RNA level, we assayed changes in *DR5* transcript levels upon activation of an inducible MYC fusion protein, MYC-ER (Eilers et al., 1989). MYC-ER is a fusion between MYC and the estrogen receptor ligand binding domain, which sequesters MYC in an inactive cytoplasmic complex unless stimulated with estrogens. Addition of the estrogen analog 4-hydroxytamoxifen (OHT) to BJ cells carrying a retrovirally encoded MYC-ER (BJ-MYCER cells) sensitized them to DR5-induced apoptosis (Supplemental Figure S3 at http://www.cancercell.org/cgi/content/full/5/5/501/DC1) and resulted in induction of *DR5* transcript within 1 hr of OHT addition, as determined by semiquantitative RT-PCR (Figure 4C), demonstrating that MYC induces *DR5* at the RNA transcript level. Addition of OHT in BJ cells lacking MYC-ER did not result in *DR5* induction.

To determine whether MYC directly induces *DR5* transcription, we activated MYC-ER in the presence of the protein synthesis inhibitor cycloheximide (CHX). Whereas CHX did not prevent induction of the well-characterized direct MYC target gene *cyclin D2* (Bouchard et al., 1999), it completely prevented *DR5* induction by MYC-ER (Figure 4C), suggesting that activation of *DR5* by MYC occurs via an indirect mechanism. This conclusion is supported by the absence of canonical CA(C/T)GTG MYC binding sites (E-boxes) in the *DR5* promoter (Yoshida et al., 2001).

To determine the functional significance of DR5 receptor upregulation by MYC, we manipulated DR5 receptor levels by siRNA-mediated gene knockdown. Although in general, siRNA-mediated gene silencing was not as effective in primary cells as in established tumor cell lines (data not shown), we were able to identify an siRNA directed against DR5 that was sufficient to knock down DR5 receptor levels on BJ-MYC cells to levels seen on BJ cells (Figure 4D). In the presence of DR5 siRNA, BJ-MYC cells were significantly protected from DR5-A (Figure 4E), arguing that sensitivity to DR5-A induced by MYC was mediated at least in part by the upregulation of DR5.

Although these results indicate that DR5 upregulation plays a vital role in MYC-induced sensitization to apoptosis, it is likely that MYC also sensitizes by other mechanisms (Juin et al., 1999; Klefstrom et al., 2002). This contention is supported by the fact that reducing DR5 levels using siRNA only partially protected BJ-MYC cells from DR5-A and that BJ-MYC cells are also susceptible to FAS-induced apoptosis if additionally sensitized by serum starvation (Supplemental Figure S4 at http://www.cancercell.org/cgi/content/full/5/5/501/DC1). However, since MADMYC was unable to inhibit tBid-induced apoptosis (Figure 3E), any additional mechanisms likely act upstream of tBid. Procaspase-8 and full-length Bid lie upstream of tBid in the death receptor pathway and show elevated protein levels in MYC-expressing cells (Figure 3B; see also Fernandez et al., 2003), raising the possibility that increased expression of these components also contributes to MYC-induced sensitization. Both *bid* and *caspase-8* contain canonical E-box motifs within their promoter regions (Fernandez et al., 2003, and not shown) and are activated upon induction of MYC-ER, even in the absence of de novo protein synthesis (Figure 4C), suggesting that they are direct transcriptional target genes of MYC (Fernandez et al., 2003).

DR5 agonists specifically kill MYC-expressing tumors in vitro and in vivo

Hahn, Weinberg, and colleagues have demonstrated that a combination of three genetic elements is sufficient to fully convert a variety of human cell types from normalcy to malignancy. These elements include *TERT*, *SV40 ER* (the early region of DNA tumor virus SV40), and an activated allele of the proto-oncogene *HRAS*, *HRAS-V12* (Elenbaas et al., 2001; Hahn et al., 1999). *SV40 ER* encodes two proteins: one, SV40 LT, disrupts the cell's two principal tumor suppressor pathways, the p53 and pRB pathways, while the other, the small t antigen, contributes to tumorigenic conversion by interacting with protein phosphatase 2A (Hahn et al., 2002).

To determine if *MYC* activation is able to confer sensitivity to DR5 signaling in the context of a tumorigenic cell, we examined human BJ fibroblasts and HA1E embryonic kidney epithelial cells that had been systematically transformed from normalcy to malignancy by the addition of these genetic elements. The fully transformed versions of each cell type by these elements (named BJELR and HA1ER, respectively) were largely insensitive to TRAIL and DR5-A (Figure 5A). However, upon introduction of an HA epitope-tagged *MYC* allele, *MYCHA*, these cells became strongly sensitized. These results demonstrate that within the cellular context of a transformed cell, as in normal cells, MYC overexpression sensitizes to DR5-mediated apoptotic signaling.

The introduction of TERT, SV40 ER, and HRAS-V12 into normal human cells renders them tumorigenic in immune-compromised mice (Elenbaas et al., 2001; Hahn et al., 1999). These cells therefore provided us with the opportunity to test MYC's ability to confer sensitivity to DR5-induced apoptosis in tumors growing in vivo, using completely genetically defined cells. Fully transformed BJELR cells, containing either MYCHA-expressing retrovirus (BJELR-MYCHA cells) or vector control retrovirus (BJELR-EGFP cells), gave rise to tumors with a latency of ap-

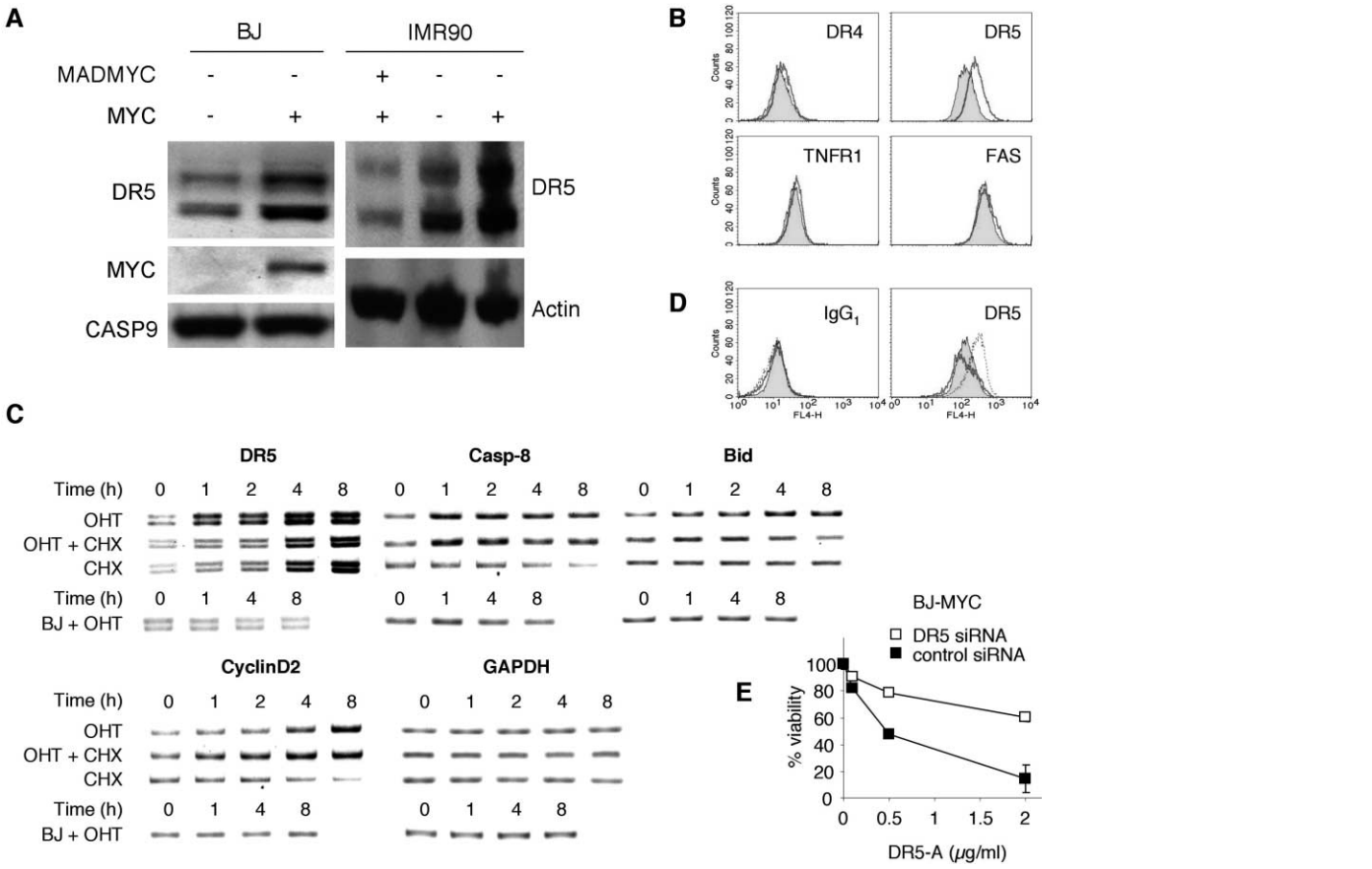

Figure 4. MYC upregulates the DR5 receptor

A: Immunoblots showing upregulation of DR5 protein levels by MYC. Caspase-9 or Actin served as a loading control.
B: Flow cytometry analysis of death receptors on the surface of intact BJ (shaded area) and BJ-MYC (open area) cells. All analyses were performed using mouse mAbs, detected with allophycocyanin-labeled goat-anti-mouse secondary antibody (Molecular Probes). DR4-A and DR5-A were used to measure DR4 and DR5 receptor cell surface levels. Similar results were obtained using HS101 and HS201 mAbs. Other antibodies used were ZB4 (anti-FAS, Upstate), MAB225 (anti-TNFR1, R&D Systems).
C: Semiquantitative RT-PCR analysis of transcript levels in BJ-MYCER cells in response to OHT, in the presence or absence of de novo protein synthesis. BJ cells lacking MYC-ER are shown as controls. Typical results from multiple independent experiments and RT-PCR analyses are shown.
D: DR5 receptor levels on BJ cells (shaded area) and BJ-MYC cells transfected with siRNA against DR5 (solid line, unshaded) or control siRNA against luciferase (dotted line, unshaded). The irrelevant mAb, MOPC21 (Sigma) was used as an IgG_1 isotype control.
E: siRNA-mediated knockdown of DR5 receptor levels desensitizes BJ-MYC to DR5-A.

proximately 4 weeks, irrespective of MYC status, upon subcutaneous injection in immune-deficient SCID-BEIGE mice. To determine the sensitivity of these tumors to DR5 signaling in vivo, we injected tumor-bearing mice intraperitoneally (IP) every second day with 100 μg of DR5-A, for a total of 5 treatments. Upon completion of the treatments, 100% (8/8) of BJELR-MYCHA tumors had responded to DR5-A treatment, showing a net reduction in tumor volume ($p = 0.006$, Figures 5B and 5C). 50% (4/8) of these were no longer detectable after treatment, either visibly or by palpation. 38% (3/8) resulted in a complete response with no signs of relapse within 1 month of treatment cessation (Figure 5D). In contrast, BJELR-EGFP-derived tumors lacking ectopic MYC did not show a statistically significant response to DR5-A ($p = 0.16$), and none of the tumors showed a net reduction in volume after treatment (0/5). Thus, despite having no significant effect on tumor latency or growth rate, ectopic MYC expression was highly significant in determining treatment outcome ($p = 0.0004$).

To determine if the efficacy of DR5-A against MYC-expressing tumors correlated with induction of apoptosis, we assayed caspase activity in tumors harvested from mice 8 hr after treatment with a single IP injection of DR5-A. BJELR-MYCHA, but not BJELR-EGFP tumors, showed strongly increased effector caspase activity in response to DR5-A (Figure 5E). This dependence upon both MYC and DR5-A for induction of caspase activity could also be observed in tumors derived from the tumorigenic kidney epithelial cell line HA1ER (Figure 5E). These results thus demonstrate, using genetically defined cells that differ only in their MYC status, that DR5 agonists can be used to induce apoptosis specifically in MYC-overexpressing tumors in vivo.

MYC activity is required for DR5-induced apoptosis in tumor-derived cell lines

The above results demonstrate the pharmacogenetic relationship between MYC and DR5 agonists in genetically engineered

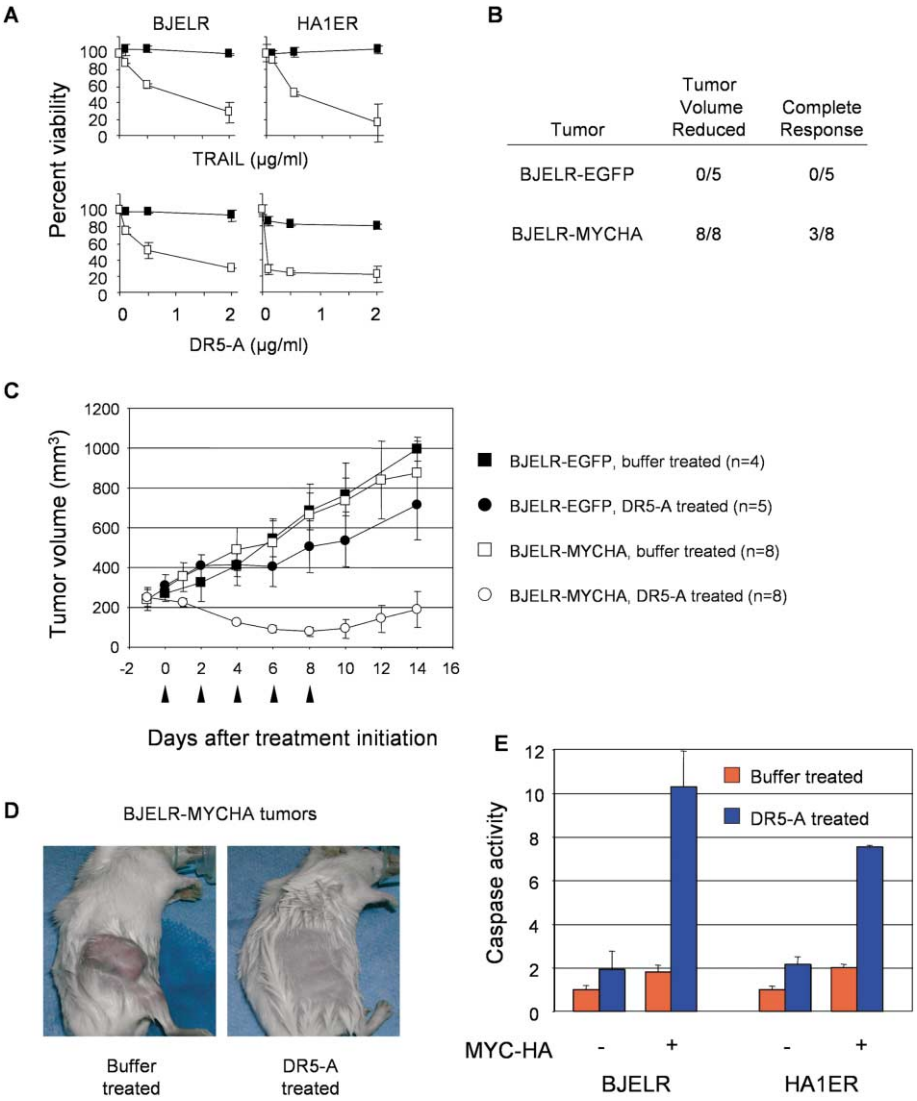

Figure 5. DR5 agonists specifically kill MYC-expressing tumors in vitro and in vivo

A: Genetically defined tumor cells BJELR and HA1ER infected with MYCHA retrovirus (open square) or EGFP control retrovirus (closed square) were assayed for sensitivity to TRAIL or DR5-A in vitro.

B and C: Response of SCID-BEIGE mice bearing BJELR-EGFP or BJELR-MYCHA-derived tumors to DR5-A treatment. Mice were IP injected with 100 μg DR5-A every other day with a total of 5 treatments, beginning when tumors reached a mean volume of 250–300 mm^3. Tumor volume was measured by caliper. Complete response was defined as elimination of visibly detectable and palpable tumors, with no relapse within 1 month following treatment completion. Filled triangles indicate DR5-A injections.

D: Representative photographs of mice bearing BJELR-MYCHA-derived tumors following 5 treatments with DR5-A or vehicle control. Example in right panel is of a mouse showing a complete response.

E: In vivo caspase activity assays. Mice carrying tumors derived from BJELR or HA1ER cells expressing MYCHA or vector control were treated with a single DR5-A (50 μg) or vehicle control treatment. Tumors were excised 8 hr post treatment, lysed, normalized for protein concentration, and assayed for caspase activity. Activities were normalized to untreated control tumors. Each group was comprised of 3 (BJELR) or 2 (HA1ER) mice.

tumor cells. To determine if a similar relationship exists in naturally arising human tumors, we introduced the dominant-negative *MADMYC* allele into a number of TRAIL-sensitive human tumor cell lines, including a cervical carcinoma (HeLa) and two colorectal carcinomas (HCT116, HCT15). Each of the cell lines expressed MYC at levels comparable to IMR90-MYC and WI38-MYC cells (Figure 6A). In each of the tumor cell lines, *MADMYC* protected significantly against DR5-A treatment, with cell survival being greater than 90% (Figure 6B). Similar protection could also be observed when MYC activity was reduced using siRNA-mediated gene knockdown (Figure 6C). Resistance was unlikely to be due to decreased proliferation caused by reduced MYC activity, because introduction of the cyclin-dependent kinase inhibitor $p16^{INK4a}$, which caused equal or more severe decreases in proliferation (Supplemental Figure S5 at http://www.cancercell.org/cgi/content/full/5/5/501/DC1), did not reduce sensitivity to DR5-A (Figure 6B). MYC activity is thus critical for sensitivity to DR5-induced apoptotic signaling in a variety of tumor cell lines.

Further characterization of one of the cell lines tested, HCT116, demonstrated that inhibition of DR5-induced cell death by MADMYC was accompanied by decreased caspase-8 processing (Figure 6D) and effector caspase activity (Figure 6E). This suggests that MYC is required at the same step in the apoptotic cascade in tumor-derived cells as in genetically defined tumorigenic cells, i.e., at the level of caspase-8 activation. To confirm that MYC activity was required for caspase-8 autocatalytic processing at the DR5 DISC, rather than for stimulation of a mitochondrial feedback loop (see Figure 3A), we examined apoptotic signaling in a $Bax^{-/-}$ derivative of HCT116 (Zhang et al., 2000). The *Bax* deficiency incapacitates the mitochondrial pathway and thus dramatically inhibits induction of effector caspase activity (Figure 6E) and cell death (Figure 6B, center) by DR5-A in these cells. Even in the absence of mitochondrial signaling, DR5-A strongly stimulated caspase-8 processing, which could be impaired by *MADMYC* (Figure 6D). Thus, in cells derived from naturally arising tumors, as in genetically defined cells, MYC activity is critical for DR5-induced apoptosis because it is required for efficient caspase-8 activation at the DR5 DISC.

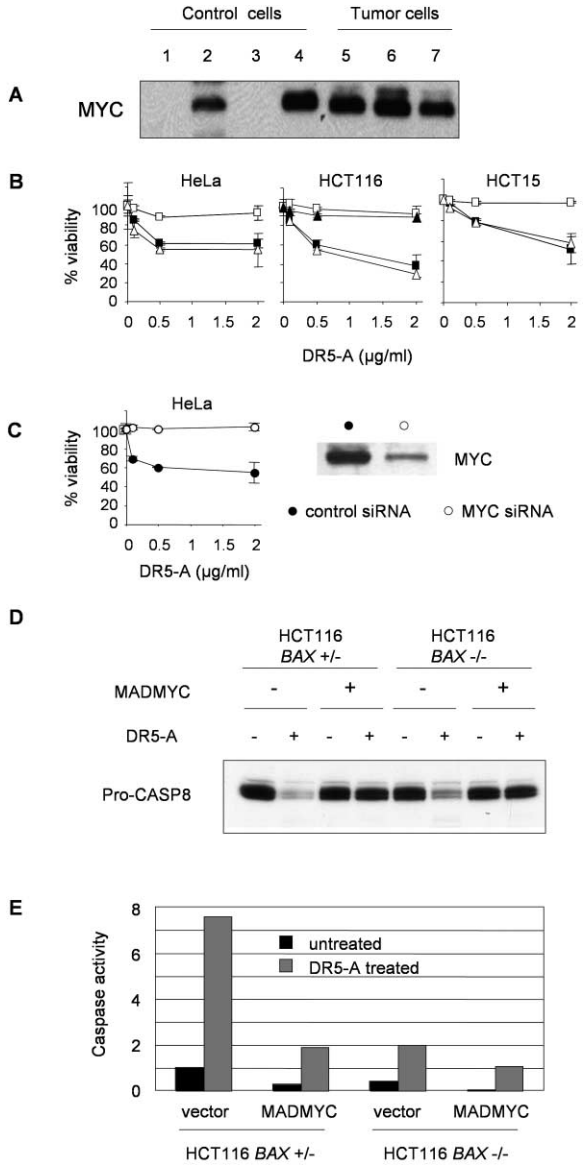

Figure 6. MYC activity is required for DR5-induced apoptosis in tumor-derived cell lines

A: Immunoblot showing MYC protein levels in normal human fibroblasts and naturally arising tumor cell lines. Lane 1, IMR90; 2, IMR90-MYC; 3, WI38; 4, WI38-MYC; 5, HeLa (cervical carcinoma); 6, HCT116 (colorectal carcinoma); 7, HCT15 (colorectal carcinoma).

B: The dominant-negative *MYC* allele, *MADMYC*, protects multiple tumor cell lines from DR5-A. Tumor cell lines were infected with control retrovirus (closed square) or pBABE-MADMYC retrovirus (open square). Open triangles represent tumor cells infected with pBABE-p16^{INK4a} retrovirus as a proliferation control and are indistinguishable from vector control cells. Filled triangles in middle panel represent *Bax*$^{-/-}$ derivatives of HCT116.

C: siRNA-mediated knockdown of MYC protects HeLa cells from DR5-A. Immunoblot shows MYC protein levels 48 hr after siRNA transfection.

D: Immunoblot showing inhibition of caspase-8 processing by MADMYC, even when mitochondrial apoptotic function is disrupted by Bax deficiency.

E: Effector caspase (DEVDase) activity assays. Values were normalized to untreated control cells.

MYC-induced sensitivity to DR5 agonists is p53 independent

DNA-damaging agents are capable of sensitizing cells to TRAIL-induced apoptosis by upregulating DR5 in a p53-dependent fashion (Wu et al., 1997). This suggests that under some circumstances, TRAIL-mediated cytotoxicity can be p53 dependent (Wang and El-Deiry, 2003). Given that MYC upregulates DR5 levels via an indirect mechanism (Figure 4C), we sought to determine if MYC's effects required functional p53 by utilizing a derivative of HCT116 in which both p53 alleles have been knocked out by homologous recombination (HCT116 p53$^{-/-}$; Bunz et al., 1999). HCT116 p53$^{-/-}$ and its isogenic p53$^{+/+}$ parent display equivalent sensitivity to DR5-A, and in each cell line, this sensitivity is MYC dependent, as apoptosis could be suppressed by overexpression of MADMYC (Figure 7A). Furthermore, surface levels of DR5 receptor were similarly downregulated by MADMYC in both cell lines (Figure 7B), demonstrating that both MYC-dependent susceptibility to DR5-induced apoptosis and upregulation of DR5 receptor do not require p53 activity.

Further evidence in support of this conclusion was obtained using a dominant-negative tumor-associated allele of *p53*, *p53-H175*. Expression of p53-H175 was sufficient to bypass γ irradiation-induced cell cycle arrest (Supplemental Figure S6 at http://www.cancercell.org/cgi/content/full/5/5/501/DC1). However, it did not abolish DR5 receptor upregulation or sensitization to DR5-A in IMR90 cells (Figures 7C and 7D) or in BJ and WI-38 cells (data not shown). Experiments are currently in progress to determine the p53-independent mechanisms by which MYC induces DR5 and sensitizes to DR5-mediated apoptosis.

Discussion

Synthetic lethal targeting of *MYC* as a therapeutic strategy

Because of its potent oncogenic activity and widespread deregulation in tumors, MYC has long made a tempting target for anticancer drug development. However, attempts to chemically disrupt its function have met with limited success, due to the inherent difficulty of inhibiting transcription factors with small molecules. Here, we demonstrate and validate an alternative, pharmacogenetic approach to targeting *MYC* based on the concept of synthetic lethality, showing that agonists of the death receptor DR5 can be used to specifically eradicate MYC-overexpressing tumors both in vitro and in vivo.

As a conceptual framework for cancer drug discovery, synthetic lethality has a number of attractive features, particularly in an era in which tumor-specific genetic alterations can be rapidly identified. First, as alluded to above, it does not require that the targeted oncogene be amenable to pharmacological targeting. In fact, the biochemical function encoded by the oncogene need not even be known, since oncoprotein function is not targeted directly by the pharmacological agent. Thus, MYC is targetable, despite being a member of a class of proteins that has been particularly intractable to chemical inhibition.

Second, the oncogene being targeted need not be essential for tumor maintenance, or even causal for tumor formation. In a number of mouse tumor models, MYC activity has been shown to be continuously required for tumor maintenance, suggesting that directly targeting MYC function may be an effective therapeutic strategy (Felsher, 2003). However, it is clear that in a

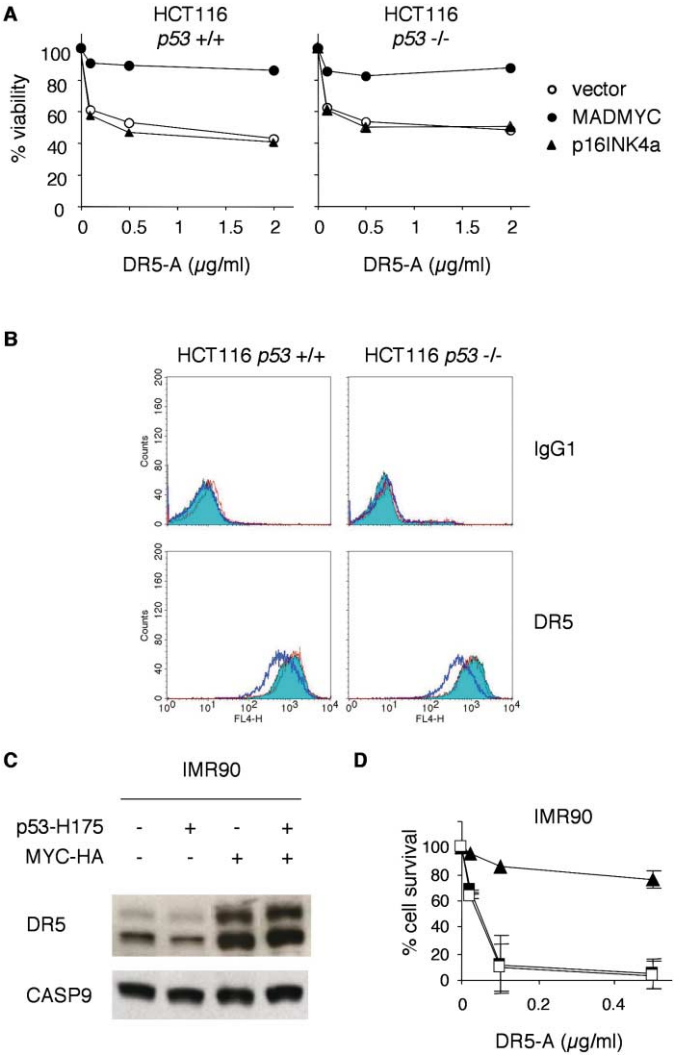

Figure 7. MYC-dependent upregulation of DR5 and sensitivity to DR5-mediated apoptosis are p53 independent

A: p53 status does not affect the MYC-dependent sensitivity of HCT116 cells to DR5-A. MYC activity was manipulated by retroviral MADMYC expression. Retroviral $p16^{INK4a}$ was used to control for proliferation.

B: Flow cytometry analysis of DR5 receptor levels on the surface of $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells. Dark blue lines represent cells overexpressing MADMYC. Light blue shaded area represents vector control cells. Thin red line represents $p16^{INK4}$-overexpressing cells and is indistinguishable from vector control cells.

C: Immunoblot showing that MYC-induced upregulation of DR5 occurs in IMR90 cells even in the presence of the dominant-negative p53 allele, p53-H175. Caspase-9 levels served as a loading control.

D: MYC-induced sensitization to DR5-A in primary cells is independent of p53. IMR90-MYC cells were infected with retrovirus expressing p53-H175 and assayed for sensitivity to DR5-A. IMR90-MYC cells (open square) and IMR90-MYC + p53-H175 cells (closed square) show indistinguishable sensitivities to DR5-A, and thus overlapping dose-response curves. Closed triangle, IMR90 + p53-H175.

subset of tumors, this dependence can be lost during the course of tumor progression, giving rise to tumors that no longer require MYC expression for their continued growth and survival (D'Cruz et al., 2001; Karlsson et al., 2003). Directly targeting MYC activity in such tumors would likely be ineffective. Since in our experimental system, control BJELR-EGFP cells formed tumors with equal efficiency and latency as BJELR-MYCHA, MYC deregulation was clearly not a causal prerequisite for tumor formation and may not have been essential for tumor maintenance. Yet MYC-expressing tumors could still be effectively targeted. Thus, synthetic lethal targeting of MYC may be an effective strategy against tumor types that frequently overexpress MYC but for which no essential role for MYC in tumor formation or maintenance has been established (Oster et al., 2002).

Third, pharmacogenetic synthetic lethality has the potential to be used as an experimental approach to uncover novel or unappreciated functions of oncogenes, in much the same manner that synthetic lethal genetic interactions have been used to elucidate biochemical or developmental pathways. Accordingly, we have used this approach to uncover a novel mechanism by which MYC sensitizes human cells to apoptosis, by upregulation of the DR5 death receptor and stimulation of caspase-8 activity. Given these advantages, it appears likely that the pharmacogenetic synthetic lethal strategy will provide a valuable complementary approach to more conventional oncogene targeting in cancer drug discovery (Reddy and Kaelin, 2002).

Bypassing tumor cells' ability to evade apoptosis

The ability to evade apoptosis is believed to be an obligatory attribute acquired during the course of tumor progression (Hanahan and Weinberg, 2000). Since MYC has been reported to sensitize cells to many different apoptotic stimuli (Hueber et al., 1997; Klefstrom et al., 1997; Pelengaris et al., 2002), under some circumstances, tumors that overexpress MYC may acquire broad resistance to apoptotic stimuli, and thus fail to respond to DR5 agonists. This appears to be the case in small cell lung carcinomas (Shivapurkar et al., 2002) and neuroblastomas (Teitz et al., 2000), in which amplification of *MYC* or its homolog *MYCN* correlates with mutation or silencing of various DISC component-encoding genes, particularly *Casp-8*. Thus, in these tumor types, MYC overexpression actually inversely correlates with TRAIL sensitivity. This has been referred to as "the N-Myc paradox" (van Noesel et al., 2003), and it suggests that DR5 agonists alone are unlikely to be effective against all tumors with deregulated MYC. However, it is clear that this scenario does not apply to all tumor types, since the human tumor-derived cell lines we tested all displayed elevated MYC levels (Figure 6A), yet showed MYC-dependent sensitivity to DR5-A (Figure 6B). In human tumors, resistance to apoptotic stimuli is more frequently achieved through inactivation of the p53 tumor suppressor pathway than by loss of DISC components. Given that the synthetic lethal interaction between DR5-A and MYC is independent of p53 status (Figure 7), DR5 agonists may be therapeutically effective against many MYC-overexpressing tumors, including those carrying p53 mutations. Indeed, Epstein Barr Virus (EBV) negative Burkitt's lymphoma cell lines, which are characterized by MYC translocations and inactivation of the p53 pathway (Lindstrom and Wiman, 2002), consistently express cell surface DR5 levels and are characteristically sensitive to TRAIL-induced apoptosis (Mouzakiti and Packham, 2003).

That DR5 agonists might be successfully used to eliminate MYC-positive tumors in human patients is perhaps most strikingly illustrated by the in vivo response of BJELR-MYCHA-derived tumors to DR5-A treatment. These tumors, which have severely impaired p53 function due to inactivation by SV40 LT, by definition contain the genetic alterations necessary to resist

the apoptotic stimuli encountered during tumorigenesis, at least in an immune-deficient animal. Yet despite their ability to evade apoptosis, they remain exquisitely sensitive to DR5 stimulation (Figure 5). While subcutaneous xenograft tumor models have their limitations as predictors of clinical outcome—they typically grow much more rapidly than spontaneously arising human tumors, and thus often overestimate the ultimate clinical effects of cytotoxic treatments (Sager and Lengauer, 2003)—the fact that isogenic BJELR-EGFP tumors lacking ectopic MYC grow in vivo at the same rate as BJELR-MYCHA, yet are insensitive to DR5-A (Figure 5C), argues that it is the pharmacogenetic and mechanistic interaction between DR5-A and *MYC*, rather than the rapid tumor growth rate, that underlies the observed treatment efficacy.

MYC: A critical determinant of TRAIL's tumor specificity

Tumor cells can become resistant to TRAIL by a number of distinct mechanisms (Ozoren and El-Deiry, 2003). As a result, comparisons between TRAIL-sensitive and -resistant tumor cell lines have provided little insight into the long unanswered question of why TRAIL sensitivity frequently arises during the course of tumorigenesis. Using defined genetic elements to systematically convert human primary cells from normalcy to malignancy, we have dissociated MYC expression and sensitivity to DR5-mediated apoptotic signaling from the process of transformation. We thereby demonstrate that DR5-mediated TRAIL sensitivity is acquired not as a necessary physiological consequence of transformation per se, but rather as a consequence of specific aberrations, such as MYC deregulation, that are frequently associated with tumor formation or progression, but absent from BJELR and HA1ER tumors.

Although in genetically defined cells, MYC overexpression is both necessary and sufficient to confer sensitivity to DR5 agonists, it remains possible that in human tumors, other genetic defects may also contribute to DR5 sensitivity. In fact, it has been proposed that tumors are primed to respond to many different apoptotic stimuli, including TRAIL signaling, by the cumulative burden of their oncogenic load (Green and Evan, 2002). In accordance with this hypothesis, it has been shown that ectopic expression of proliferation-promoting genes such as *E2F1* can also sensitize human cells to death ligands, including TRAIL (Phillips et al., 1999) and DR5-A (Y.W. and K.C.Q., unpublished data), albeit moderately. However, E2F1 is unlikely to play a major role in determining sensitivity to DR5 agonists in human tumors. In human cancer, E2F activity is most frequently deregulated by loss of one of its upstream negative regulators, $p16^{INK4a}$, and to a lesser extent pRB, yet inactivation of pRB function by SV40 LT failed to sensitize either HA1ER or BJELR cells to DR5-A or TRAIL, in vitro or in vivo (Figure 5). Furthermore, re-introduction of $p16^{INK4a}$ into 2 tumor cell lines that lack its expression, HCT116 and HCT15, inhibited proliferation (Supplemental Figure S5 at http://www.cancercell.org/cgi/content/full/5/5/501/DC1), presumably by attenuating E2F activity, yet had no effect on sensitivity to DR5-A (Figure 6B). In striking contrast, reducing MYC activity severely reduced sensitivity (Figures 6B and 6C), suggesting that MYC, but not E2F activity, plays a major and critical role in determining the sensitivity of these tumor cells to DR5 agonists.

The results presented here identify a synthetic lethal interaction between DR5 agonists and *MYC* and elucidate the mechanistic basis for this pharmacogenetic relationship. Importantly, they demonstrate that this interaction can be therapeutically exploited to eradicate tumors in a genotype-selective fashion in an animal model and thus validate DR5 agonists, as well as the strategy of pharmacogenetic synthetic lethality, as feasible approaches to targeting MYC in human cancer. With the ongoing development of DR5 agonists as promising anticancer drugs, MYC overexpression and its associated sensitization to apoptosis may prove to be an exploitable Achilles heel for many human cancers.

Experimental procedures

Cell lines

Normal human fibroblasts BJ, IMR90, and WI38 and tumor cell lines HeLa, HCT116, and HCT15 were obtained from the American Type Culture Collection (ATCC). $Bax^{-/-}$ and $Bax^{+/-}$ derivatives of HCT116 were obtained from Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Genetically defined immortalized or transformed cells HA1E, HA1ER, BJELB, and BJELR were obtained from Robert Weinberg (Whitehead Institute, Cambridge, MA). Tumor cell lines were grown as recommended by ATCC. IMR90 and WI38 were grown in Minimum Essential Medium Eagle (ATCC) supplemented with glutamine, penicillin/streptomycin, and 10% heat inactivated fetal bovine serum (FBS). HA1E-derived cells were grown in MEM-α medium supplemented as above. BJ-derived cells were grown in a mixture of DMEM/Medium199 at a 4:1 ratio, supplemented as above but with 15% FBS. All cells were grown at 37°C in a 5% CO_2 humidified incubator.

Retroviruses

Retroviruses were produced by Lipofectamine 2000 (Invitrogen)-mediated transfection into Phoenix-A producer cells (Garry Nolan, Stanford University, Stanford, CA). Retroviral infections were performed by centrifuging target cells at 2700 rpm for 60–90 min at 25°C with 50% retroviral supernatant containing 20 mM HEPES and 8 μg/ml polybrene. Typically, 2–3 rounds of infection were performed to ensure infection of more than 98% of cells, as determined by flow cytometry. Retroviruses used were derivatives of pBABEpuro, pWZLhygro, and LZRS-IRES-EGFP. pBABEpuro was used for MYC, HRAS-V12, p53-H175, MADMYC, TERT, Bcl-xL, $p27^{KIP1}$, and $p16^{INK4a}$ retroviruses; pWZLhygro was used for p53-H175 retrovirus; and LZRS-IRES-EGFP was used for MYCHA retrovirus. 2 μg/ml puromycin or 200 μg/ml hygromycin was used for selection.

Antibodies

Antibodies used for immunoblotting were obtained from the indicated suppliers: MYC and Actin (Santa Cruz Biotechnology), caspase-8 and Bid (Becton Dickinson), DR5 (Cayman Chemical Company), caspase-9 (Cell Signaling), and caspase-3 (R&D Systems). Anti-FAS functional antibody used was purified IgM from clone CH11 (MBL International). DR4-A and DR5-A were mouse IgG_1 mAbs and will be described in greater detail elsewhere (D.A.K. and M.N., unpublished data). For in vitro apoptosis assays, these mAbs were crosslinked by incubating them for 1 hr at room temperature, at a 1:3 ratio by weight with $F(Ab')_2$ fragment goat anti-mouse anti-Fcγ (Jackson ImmunoResearch).

Cell death assays

Three thousand to ten thousand cells per well were plated in 96-well plates in the appropriate cell culture medium, allowed to attach overnight, and treated with various apoptotic stimuli for 16 hr. TRAIL preparations used: Figure 1 and Supplemental Figure S4, Super *Killer* TRAIL (Alexis Corp), comprised of the C-terminal extracellular domain of human TRAIL (residues 95–281) fused at its N terminus to a His-tag; Figures 2 and 6, TRAIL C-terminal fragment (residues 114–281, Calbiochem). Where concentrations are not indicated, the following doses were used: Super *Killer* TRAIL (200 ng/ml), TNFα (100 ng/ml), CH11 anti-FAS (2 μg/ml), γ-irradiation (20 Gy), cisplatin (20 μM), etoposide (50 μM). Cell viability assays were performed

in triplicate using CellTiter-Glo (Promega) according to the manufacturer's instructions.

Transient transfections

HA1E-MYC or BJELB-MYC (BJ-MYC cells immortalized with SV40 LT and TERT) cells were transfected with pCMV-MADMYC in 384-well plates using Fugene 6 transfection reagent (Roche). Apoptosis was induced by cotransfection with pCDNA3-tBid in the indicated amounts, or by adding 2 μg/ml crosslinked DR5-A antibody 48 hr posttransfection. Viability of the successfully transfected cell population was measured 64 hr posttransfection by assaying luciferase activity from a cotransfected CMV-luciferase reporter construct (50 ng/well) using Bright-Glo (Promega). In all experiments, empty vector was used to adjust the total amount of DNA transfected to 100 ng, if necessary. Assays were performed in triplicate. Transfection efficiencies were typically 30%–60%.

Semiquantitative RT-PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen). 1 μg RNA was reverse transcribed using ThermalScript System (Invitrogen). 1 μl cDNA was then used for PCR amplification. The number of PCR cycles was determined empirically to ensure that amplification was halted during the exponential phase. 1 μM OHT was used for induction of MYC-ER. 50 μg/ml CHX was added to block protein synthesis where indicated. When both 4-OHT and CHX were added to the cells, CHX was added a few minutes before 4-OHT.

siRNA

All siRNAs were purchased from Dharmacon. MYC siRNA was a pool of 4 siRNAs of proprietary sequence (SMARTpool). 3 individual MYC siRNA's gave similar results to the SMARTpool (data not shown). Sequences of these and other siRNAs used were: siMYC1 AAG AUG AGG AAG AAA UCG AUG UU; siMYC4 AAA AGG UCA GAG UCU GGA UCA CC; siMYC5 CAC GUC UCC ACA CAU CAG CAC AA; siDR5 AAA UGA GAU AAA GGU GGC UAA UU; control siRNA siGL3 (directed against luciferase from vector pGL3) AAC UUA CGC UGA GUA CUU CGA UU. Cells were transfected with 200 ng siRNA per well in 12-well plates using Lipofectamine 2000 (Invitrogen). Cells were treated with DR5-A or prepared for FACS analysis 48 hr after transfection.

Animal experiments

5×10^6 BJELR-MYCHA or BJELR-EGFP tumorigenic cells were resuspended in 100 μl 60% Matrigel (BD Biosciences) and injected subcutaneously into the flank of 6- to 8-week-old male SCID-BEIGE mice (Taconic). Tumors arose in 100% of animals with a mean latency of 4 weeks. Tumors were measured with calipers in two dimensions, and volumes were calculated based on the formula for an oblate spheroid, $V = 1/6\ \pi(\text{length})(\text{width})^2$. When tumors reached a mean volume of 250–300 mm^3, mice were rank-ordered by tumor volume with the smallest and largest tumor excluded, and then divided into even-rank and odd-rank groups for IP injection with DR5-A or 200 μl vehicle control (50 mM sodium citrate [pH 7.0], 140 mM NaCl). For in vivo caspase activity assays, BJELR-derived tumorigenic cells were injected into mice as above; HA1ER cells were injected without Matrigel into athymic NCr nu/nu mice (Simonsen Laboratories). Tumors were surgically excised 8 hr after treatment, homogenized in 300 μl PBS with a tissue homogenizer, and lysed by addition of Triton X-100 to 1% final concentration. Clarified supernatants were normalized for protein and assayed for caspase-3 activity using Ac-DEVD-AFC (Calbiochem) as substrate as previously described (Deveraux et al., 2000). All animal husbandry and experiments were performed in accordance with protocols approved by the IACUC committee of the Genomics Institute of the Novartis Research Foundation.

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References

Ashkenazi, A. (2002). Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat. Rev. Cancer *2*, 420–430.

Berns, K., Hijmans, E.M., and Bernards, R. (1997). Repression of c-Myc responsive genes in cycling cells causes G1 arrest through reduction of cyclin E/CDK2 kinase activity. Oncogene *15*, 1347–1356.

Boatright, K.M., Renatus, M., Scott, F.L., Sperandio, S., Shin, H., Pedersen, I.M., Ricci, J.E., Edris, W.A., Sutherlin, D.P., Green, D.R., and Salvesen, G.S. (2003). A unified model for apical caspase activation. Mol. Cell *11*, 529–541.

Bouchard, C., Thieke, K., Maier, A., Saffrich, R., Hanley-Hyde, J., Ansorge, W., Reed, S., Sicinski, P., Bartek, J., and Eilers, M. (1999). Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. EMBO J. *18*, 5321–5333.

Bunz, F., Hwang, P.M., Torrance, C., Waldman, T., Zhang, Y., Dillehay, L., Williams, J., Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1999). Disruption of p53 in human cancer cells alters the responses to therapeutic agents. J. Clin. Invest. *104*, 263–269.

Capdeville, R., Buchdunger, E., Zimmermann, J., and Matter, A. (2002). Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. Nat. Rev. Drug Discov. *1*, 493–502.

D'Cruz, C.M., Gunther, E.J., Boxer, R.B., Hartman, J.L., Sintasath, L., Moody, S.E., Cox, J.D., Ha, S.I., Belka, G.K., Golant, A., et al. (2001). c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous Kras2 mutations. Nat. Med. *7*, 235–239.

Deveraux, Q.L., Welsh, K., and Reed, J.C. (2000). Purification and use of recombinant inhibitor of apoptosis proteins as caspase inhibitors. Methods Enzymol. *322*, 154–161.

Dolma, S., Lessnick, S.L., Hahn, W.C., and Stockwell, B.R. (2003). Identification of genotype-selective antitumor agents using synthetic lethal chemical screening in engineered human tumor cells. Cancer Cell *3*, 285–296.

Druker, B.J. (2002). Perspectives on the development of a molecularly targeted agent. Cancer Cell *1*, 31–36.

Eilers, M., Picard, D., Yamamoto, K.R., and Bishop, J.M. (1989). Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. Nature *340*, 66–68.

Elenbaas, B., Spirio, L., Koerner, F., Fleming, M.D., Zimonjic, D.B., Donaher, J.L., Popescu, N.C., Hahn, W.C., and Weinberg, R.A. (2001). Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev. *15*, 50–65.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z., and Hancock, D.C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. Cell *69*, 119–128.

Fantin, V.R., Berardi, M.J., Scorrano, L., Korsmeyer, S.J., and Leder, P. (2002). A novel mitochondriotoxic small molecule that selectively inhibits tumor cell growth. Cancer Cell *2*, 29–42.

Felsher, D.W. (2003). Cancer revoked: oncogenes as therapeutic targets. Nat. Rev. Cancer *3*, 375–380.

Fernandez, P.C., Frank, S.R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A., and Amati, B. (2003). Genomic targets of the human c-Myc protein. Genes Dev. *17*, 1115–1129.

Fulda, S., Meyer, E., and Debatin, K.M. (2002). Inhibition of TRAIL-induced apoptosis by Bcl-2 overexpression. Oncogene *21*, 2283–2294.

Grandori, C., Cowley, S.M., James, L.P., and Eisenman, R.N. (2000). The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu. Rev. Cell Dev. Biol. *16*, 653–699.

Green, D.R., and Evan, G.I. (2002). A matter of life and death. Cancer Cell *1*, 19–30.

Griffith, T.S., Rauch, C.T., Smolak, P.J., Waugh, J.Y., Boiani, N., Lynch, D.H., Smith, C.A., Goodwin, R.G., and Kubin, M.Z. (1999). Functional analysis of TRAIL receptors using monoclonal antibodies. J. Immunol. *162*, 2597–2605.

Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, R.A. (1999). Creation of human tumour cells with defined genetic elements. Nature *400*, 464–468.

Hahn, W.C., Dessain, S.K., Brooks, M.W., King, J.E., Elenbaas, B., Sabatini, D.M., DeCaprio, J.A., and Weinberg, R.A. (2002). Enumeration of the simian virus 40 early region elements necessary for human cell transformation. Mol. Cell. Biol. *22*, 2111–2123.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57–70.

Hartwell, L.H., Szankasi, P., Roberts, C.J., Murray, A.W., and Friend, S.H. (1997). Integrating genetic approaches into the discovery of anticancer drugs. Science *278*, 1064–1068.

Hueber, A.O., Zornig, M., Lyon, D., Suda, T., Nagata, S., and Evan, G.I. (1997). Requirement for the CD95 receptor-ligand pathway in c-Myc-induced apoptosis. Science *278*, 1305–1309.

Ichikawa, K., Liu, W., Zhao, L., Wang, Z., Liu, D., Ohtsuka, T., Zhang, H., Mountz, J.D., Koopman, W.J., Kimberly, R.P., and Zhou, T. (2001). Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. Nat. Med. *7*, 954–960.

Juin, P., Hueber, A.O., Littlewood, T., and Evan, G. (1999). c-Myc-induced sensitization to apoptosis is mediated through cytochrome c release. Genes Dev. *13*, 1367–1381.

Karlsson, A., Giuriato, S., Tang, F., Fung-Weier, J., Levan, G., and Felsher, D.W. (2003). Genomically complex lymphomas undergo sustained tumor regression upon MYC inactivation unless they acquire novel chromosomal translocations. Blood *101*, 2797–2803.

Klefstrom, J., Arighi, E., Littlewood, T., Jaattela, M., Saksela, E., Evan, G.I., and Alitalo, K. (1997). Induction of TNF-sensitive cellular phenotype by c-Myc involves p53 and impaired NF-kappaB activation. EMBO J. *16*, 7382–7392.

Klefstrom, J., Verschuren, E.W., and Evan, G. (2002). c-Myc augments the apoptotic activity of cytosolic death receptor signaling proteins by engaging the mitochondrial apoptotic pathway. J. Biol. Chem. *277*, 43224–43232.

LeBlanc, H., Lawrence, D., Varfolomeev, E., Totpal, K., Morlan, J., Schow, P., Fong, S., Schwall, R., Sinicropi, D., and Ashkenazi, A. (2002). Tumor-cell resistance to death receptor–induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. Nat. Med. *8*, 274–281.

Leverkus, M., Sprick, M.R., Wachter, T., Denk, A., Brocker, E.B., Walczak, H., and Neumann, M. (2003). TRAIL-induced apoptosis and gene induction in HaCaT keratinocytes: differential contribution of TRAIL receptors 1 and 2. J. Invest. Dermatol. *121*, 149–155.

Lindstrom, M.S., and Wiman, K.G. (2002). Role of genetic and epigenetic changes in Burkitt lymphoma. Semin. Cancer Biol. *12*, 381–387.

Mouzakiti, A., and Packham, G. (2003). Regulation of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Burkitt's lymphoma cell lines. Br. J. Haematol. *122*, 61–69.

Nesbit, C.E., Tersak, J.M., and Prochownik, E.V. (1999). MYC oncogenes and human neoplastic disease. Oncogene *18*, 3004–3016.

Oster, S.K., Ho, C.S., Soucie, E.L., and Penn, L.Z. (2002). The myc oncogene: MarvelouslY Complex. Adv. Cancer Res. *84*, 81–154.

Ozoren, N., and El-Deiry, W.S. (2003). Cell surface Death Receptor signaling in normal and cancer cells. Semin. Cancer Biol. *13*, 135–147.

Pelengaris, S., Khan, M., and Evan, G. (2002). c-MYC: more than just a matter of life and death. Nat. Rev. Cancer *2*, 764–776.

Peter, M.E. (2000). The TRAIL DISCussion: it is FADD and caspase-8! Cell Death Differ. *7*, 759–760.

Phillips, A.C., Ernst, M.K., Bates, S., Rice, N.R., and Vousden, K.H. (1999). E2F-1 potentiates cell death by blocking antiapoptotic signaling pathways. Mol. Cell *4*, 771–781.

Reddy, A., and Kaelin, W.G., Jr. (2002). Using cancer genetics to guide the selection of anticancer drug targets. Curr. Opin. Pharmacol. *2*, 366–373.

Sager, J.A., and Lengauer, C. (2003). New paradigms for cancer drug discovery. Cancer Biol. Ther. *2*, 452–455.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H., and Peter, M.E. (1998). Two CD95 (APO-1/Fas) signaling pathways. EMBO J. *17*, 1675–1687.

Shivapurkar, N., Reddy, J., Matta, H., Sathyanarayana, U.G., Huang, C.X., Toyooka, S., Minna, J.D., Chaudhary, P.M., and Gazdar, A.F. (2002). Loss of expression of death-inducing signaling complex (DISC) components in lung cancer cell lines and the influence of MYC amplification. Oncogene *21*, 8510–8514.

Smyth, M.J., Takeda, K., Hayakawa, Y., Peschon, J.J., van den Brink, M.R., and Yagita, H. (2003). Nature's TRAIL—on a path to cancer immunotherapy. Immunity *18*, 1–6.

Sprick, M.R., Rieser, E., Stahl, H., Grosse-Wilde, A., Weigand, M.A., and Walczak, H. (2002). Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. EMBO J. *21*, 4520–4530.

Teitz, T., Wei, T., Valentine, M.B., Vanin, E.F., Grenet, J., Valentine, V.A., Behm, F.G., Look, A.T., Lahti, J.M., and Kidd, V.J. (2000). Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. Nat. Med. *6*, 529–535.

Torrance, C.J., Agrawal, V., Vogelstein, B., and Kinzler, K.W. (2001). Use of isogenic human cancer cells for high-throughput screening and drug discovery. Nat. Biotechnol. *19*, 940–945.

Vafa, O., Wade, M., Kern, S., Beeche, M., Pandita, T.K., Hampton, G.M., and Wahl, G.M. (2002). c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. Mol. Cell *9*, 1031–1044.

van Noesel, M.M., Pieters, R., Voute, P.A., and Versteeg, R. (2003). The N-myc paradox: N-myc overexpression in neuroblastomas is associated with sensitivity as well as resistance to apoptosis. Cancer Lett. *197*, 165–172.

Wang, S., and El-Deiry, W.S. (2003). Requirement of p53 targets in chemosensitization of colonic carcinoma to death ligand therapy. Proc. Natl. Acad. Sci. USA *100*, 15095–15100.

Wu, G.S., Burns, T.F., McDonald, E.R., 3rd, Jiang, W., Meng, R., Krantz, I.D., Kao, G., Gan, D.D., Zhou, J.Y., Muschel, R., et al. (1997). KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat. Genet. *17*, 141–143.

Yoshida, T., Maeda, A., Tani, N., and Sakai, T. (2001). Promoter structure and transcription initiation sites of the human death receptor 5/TRAIL-R2 gene. FEBS Lett. *507*, 381–385.

Zhang, L., Yu, J., Park, B.H., Kinzler, K.W., and Vogelstein, B. (2000). Role of BAX in the apoptotic response to anticancer agents. Science *290*, 989–992.