Identification of Ras-Related Nuclear Protein, Targeting Protein for *Xenopus* Kinesin-like Protein 2, and Stearoyl-CoA Desaturase 1 as Promising Cancer Targets from an RNAi-Based Screen

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Abstract

To identify new candidate cancer drug targets, we used RNAi as a tool to functionally evaluate genes that play a role in maintaining human tumor cell survival. We screened a small interfering RNA (siRNA) library directed against ~3,700 individual genes to assess the ability of siRNAs to induce cell death in an *in vitro* cell cytotoxicity assay. We found that siRNAs specifically targeting ras-related nuclear protein (Ran), targeting protein for *Xenopus* kinesin-like protein 2 (TPX2), and stearoyl-CoA desaturase 1 (SCD1), significantly reduced the survival of multiple human tumor cell lines. Further target validation studies revealed that treatment with Ran and TPX2 siRNAs differentially reduced the survival of activated K-Ras–transformed cells compared with their normal isogenic counterparts in which the mutant *K-Ras* gene had been disrupted (DKS-8). Knockdown of Ran and TPX2 in activated mutant K-Ras cells selectively induced S-phase arrest or transient G_2-M arrest phenotypes, respectively, that preceded apoptotic cell death. Given our observations that Ran and TPX2 depletion preferentially reduces the survival of activated K-Ras–transformed cells, these two proteins may serve as useful anticancer targets in tumors expressing the activated K-Ras oncogene. [Cancer Res 2007; 67(9):4390–8]

Introduction

With the advent of large-scale gene knockdown using small interfering RNA (siRNA) libraries, it has become possible to quickly identify new cancer drug targets as well as explore their role in tumorigenesis. Currently, RNAi is being widely used in mammalian cell–based systems to probe known signal transduction pathways for the identification of novel genes. Studies using smaller siRNA libraries identified Akt-cooperating kinases (1), genes influencing tumor necrosis factor–related apoptosis–inducing ligand induced cell killing (2), novel regulators of apoptosis and chemoresistance genes (3), products involved in endoplasmic reticulum stress-dependent apoptosis (4), and a novel familial cylindromatosis tumor suppressor gene found to negatively regulate nuclear factor κB (NF-κB) signaling (5). In addition to these smaller scale libraries, siRNA technology has also rapidly expanded to include systematic, larger scale gene knockdown in mammalian cells (6, 7). Namely, retroviral-based siRNA libraries targeting about one third of the human genome successfully identified genes involved in p53-mediated cell cycle arrest (8), human proteasome function (9), and novel tumor suppressor pathways (10, 11). Generation of large siRNA libraries based on the processing of long dsRNA by *Escherichia coli* RNaseIII endoribonuclease have also been screened to successfully identify genes required for mitosis (12).

An important issue in using siRNA as a tool for genome-wide functional screening is the potential for obtaining off-target effects. Recent studies in the field suggest that siRNA specificity is not absolute, and off-target effects can be induced by several different mechanisms that include miRNA-like inhibition of translation (13, 14), induction of an IFN response (15), and global up-/down-regulation of genes arising from high concentrations of siRNA (16, 17). One of the major factors responsible for off-target effects is partial sequence cross-hybridization to untargeted transcripts (18–20). In one of these studies, a detailed analysis of the specificity of siRNA concluded that perfect matches between the 5′ end of antisense siRNA (positions 2–7 or 2–8) and the 3′ untranslated region of genes dictated the off-targeting effects (20). Taken together, these data have strong implications for data interpretation derived from large-scale siRNA library screens. To help mitigate the potential complications arising from such off-target effects, general guidelines for good practice in RNAi experiments are necessary and include correlating phenotypic outcome with siRNA-mediated knockdown using multiple siRNAs that target different regions of the same gene and using siRNA at the lowest possible effective concentration to enhance specificity (21).

To identify new cancer targets, we screened a siRNA library against 3,700 genes to assess the ability of siRNAs to induce cell death using an *in vitro* cell cytotoxicity assay. Many of the initial hits in the screen were due to off-target effects. Nevertheless, three hits were identified and confirmed with multiple siRNAs as being essential for human tumor cell survival. These proteins may serve as useful cancer targets.

Materials and Methods

Cell lines. Human non–small cell lung carcinoma cell line H1299 and human breast carcinoma cell line MDA-MB-468 (American Type Culture Collection) were cultured in RPMI 1640 (Invitrogen Corp.) supplemented with 10% fetal bovine serum (FBS, Invitrogen). Human colon cancer cell lines DLD-1 (parental line containing both mutant and wild-type K-Ras alleles), DKO-1 (containing only mutant K-Ras allele), DKS-8, DKO-3, and DKO-4 (all containing wild-type K-Ras allele) were obtained from the laboratories of Dr. Senji Shirasawa and Dr. Takehiko Sasazuki (International Medical Center of Japan, Tokyo, Japan). Cells were cultured in Dulbecco's modified medium with 10% FBS (Invitrogen). All cells were maintained at 37°C in a humidified chamber containing 5% CO_2/95% air.

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doi:10.1158/0008-5472.CAN-06-4132

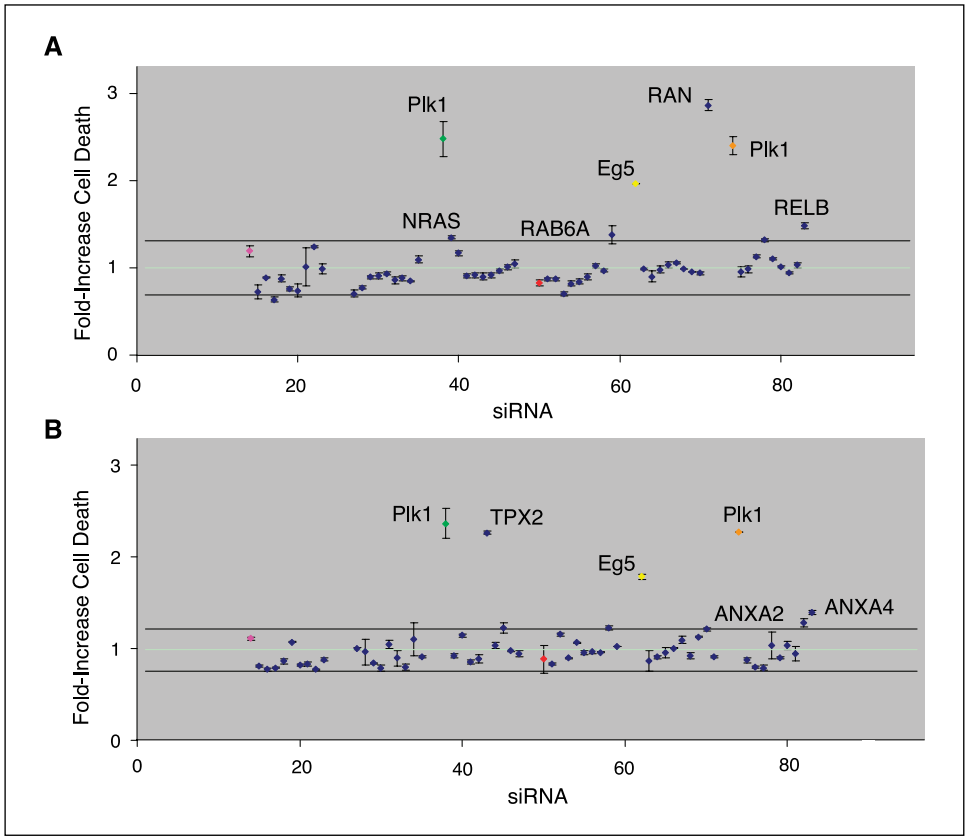

Figure 1. Screening of siRNA library in the H1299 cell line. H1299 cells were transfected with siRNA in triplicate, and 72 h later, cytotoxicity was assessed using the Toxilight assay. *A* and *B,* representative graphs of two 96-well plates from the library screen, each containing 60 siRNAs. The middle line represents the population average, and the upper and lower lines depict 1 SD above and below the average, respectively. Eg5 (*yellow*) and Plk1 (*green, orange*) siRNAs represent positive controls, and scrambled siRNAs (*pink, red*) represent negative controls.

siRNAs and siRNA library design. Our siRNA library was selected to target the druggable genome (~3,700 genes), which included protein kinases, G-protein–coupled receptors, ubiquitin E3 ligases, transporters, ion channels, peptidases, phophatases, dehydrogenases, nuclear receptors, lipid-modifying enzymes, integrins, chemokines, and receptors. The siRNAs against each gene were designed by Dharmacon Research, Inc., using their proprietary algorithm whereby each mRNA is targeted by a pool of siRNAs consisting of a combination of four siRNA duplexes directed at different regions of the gene. siRNA to the *polo-like kinase-1* (*Plk1*) gene (GenBank accession no. NM_005030) and a universal nontargeting siRNA were used as positive and negative controls, respectively. Additional siRNAs targeting ras-related nuclear protein (Ran), GenBank accession no. NM_006325, include (5′-3′) Ran-1: AGAAGAAUCUUCAGUACUAUU; Ran-2: GUGAAUUUGAGAAGAAGUAUU; Ran-3: CCUAUUAAGUUCAAUGUAUUU; and Ran-4: ACAGGAAAGUGAAGGCGAAUU. Additional siRNAs against targeting protein for *Xenopus* kinesin-like protein 2 (TPX2), GenBank accession no. NM_012112, include 5′-3′, TPX2-1: UGACAACACUUACUACAAAUU; TPX2-2: GGACGAACCGGUAGUGAUAUU; TPX2-3: AGACAAAGAACGUCAGUUAUU; and TPX2-4: GAACUUUACAUCUGAACUAUU. Additional siRNAs targeting *stearoyl-CoA desaturase 1* (SCD1), GenBank accession no. NM_005063, include 5′–3′, SCD1-1: GGAGAAACAUCAUCCUUAUUU; SCD1-2: GAUAUGCUGUGGUGCUUAAUU; SCD1-3: GAGAUAAGUUGGAGACGAUUU; and SCD1-4: AGAAUGAUGUCUA UGAAUGUU.

siRNA library screen. H1299 cells were seeded the day before siRNA transfections at ~5×10^3 cells per well in 96-well plates to obtain 50% to 60% confluency at the time of transfection. The library was screened using four siRNAs/target as a pool at a concentration of 25 nmol/L each. In brief, for a single well of a 96-well plate, 6 μL of siRNA and 3 μL of LipofectAMINE 2000 (Invitrogen Corp.) were each incubated separately with 100 μL of Opti-MEM (Invitrogen Corp.) for 10 min, mixed together for 20 min at room temperature, and then 20 μL applied to the cells plated in 100 μL of medium. The cells were incubated in the siRNA transfection reagent mixture for 4 to 5 h at 37°C before receiving fresh medium (100 μL). Three days later, cell death was measured using the Toxilight assay (Cambrex Corporation) according to the manufacturer's instructions. A Z' factor was calculated (0.38) to validate the suitability and robustness of the assay for high-throughput screening (22). A Z' factor is defined as the screening window coefficient and is reflective of both the signal dynamic range and the variability in sample data measurements. A Z' factor value that is above zero suggests an assay that is suitable for high-throughput screening (22). Raw data values were recorded as luciferase units on a 1420 VICTOR Multilabel Counter (Perkin-Elmer Life Sciences). Each siRNA transfection was done in triplicate, spanning three independent 96-well plates, such that normalized values were averaged for three plates to obtain average fold increase in cell death relative to control for each siRNA treatment.

Western blotting. Cell lysates were electrophoresed in Novex SDS-PAGE gels (Invitrogen Corp.), and the proteins were transferred onto a nitrocellulose membrane. Immunoblotting was done using a rabbit anti-Ran polyclonal antibody (Cell Signaling Technology), an anti-TPX2 antibody (Rockland Immunochemicals), a mouse anti-SCD1 monoclonal antibody (Santa Cruz Biotechnology), or a mouse anti-actin monoclonal antibody (Sigma-Aldrich Corporation). Blots were developed using the enhanced chemiluminescence (ECL) reagent from Amersham Biosciences.

Caspase-3 assay. siRNA-transfected cells in 96-well plates were lysed in 120 μL of 1× lysis buffer [1.67 mmol/L HEPES (pH 7.4), 7 mmol/L KCl, 0.83 mmol/L $MgCl_2$, 0.11 mmol/L EDTA, 0.11 mmol/L EGTA, 0.57% CHAPS, 1 mmol/L DTT, 1× protease inhibitor cocktail tablet; Roche Pharmaceuticals]. After cell lysis, 80 μL of a caspase-3 reaction buffer [48 mmol/L HEPES (pH 7.5), 252 mmol/L sucrose, 0.1% CHAPS, 4 mmol/L DTT, and 20 μmol/L Ac-DEVD-AMC substrate; Biomol Research Labs, Inc.] were added, and the plates were incubated for 2 h at 37°C. The plates were read on a 1420 VICTOR Multilabel Counter (Perkin-Elmer Life Sciences) at excitation = 360/40 and emission = 460/40. The fluorescence units relative to the control were defined as fold increase in caspase-3 activity.

Cell proliferation assay. Inhibition of cellular proliferation was measured using the ATPlite assay according to the manufacturer's

instructions (Perkin-Elmer Life Sciences). siRNA-transfected cells in 96-well plates were lysed in 100 μL of lysis buffer, and the amount of ATP levels in live cells was recorded as luciferase units on a 1420 VICTOR Multilabel Counter (Perkin-Elmer Life Sciences).

Flow cytometry analysis. Cell cycle distribution and DNA synthesis were assessed by propidium iodide labeling and 5-bromo-2-deoxyuridine (BrdUrd) incorporation, respectively. Approximately 1×10^6 cells were pulse labeled with 30 μmol/L BrdUrd (Sigma-Aldrich) in 6-well plates for 1 h at the selected times post-siRNA transfection, harvested, and then fixed in 70% methanol. Methanol-fixed cells were resuspended in 1 mL of 2 N HCl, 0.5% Triton X-100 for 30 min at 25°C, after which the suspension was neutralized with the addition of 1 mL of 0.1 mol/L sodium tetraborate (pH 8.5). Replicative DNA synthesis was detected by staining the BrdUrd-containing cells with 0.1 mL of FITC-conjugated anti-BrdUrd antibody (Becton Dickinson) for 30 min at 25°C. Cells were washed once in 1× washing buffer and resuspended in 800 μL of 1× PBS, 200 μL of propidium iodide (0.1 mg/mL), and 5 μL of RNase A (10 mg/mL; Sigma-Aldrich), incubated in the dark (25°C, 30 min), and analyzed using a Becton Dickinson ExCalibur flow cytometer.

Results

RNAi-based library screen to identify genes required for human tumor cell survival. To identify targets involved in human tumor cell proliferation, we screened a library of synthetic siRNAs using a cell death assay. Figure 1 depicts representative results from the library screen. The sample siRNA transfections resulted in a fairly tight range of data values that equated to a negligible cell death phenotype, demonstrating that our siRNA library screening method was tightly controlled. Due to the low background of our phenotypic screen, we were able to easily detect siRNAs that induced cell kill at or above 1 SD from the population mean. For example, treatment with positive control siRNAs against Plk1 and Eg5 resulted in an approximately 2-fold induction of cell death, consistent with studies demonstrating a relationship between inhibition of Plk1 and Eg5 expression and antiproliferative effects (23, 24). Examples of siRNA hits from our screen that caused a significant increase in cell death relative to the rest of the population include Ran, NRAS, RAB6A, RelB, TPX2, ANXA2, and ANXA4 (Fig. 1*A* and *B*). Based on our initial screen of the siRNA library, 48 hits were identified for further evaluation (~1.3% hit rate). Retest assessment of these hits was done using lower siRNA doses ranging from ~1.5 to 6 nmol/L to reveal that 23 out of the 48 hits were reproducible and potent at single nanomole-per-liter doses relative to a scrambled siRNA control (data not shown). Included in these target hits were Plk1 and STK6 (Aurora A), thus demonstrating the ability of our screen to detect hits that have been well established as playing a role in tumorigenesis.

siRNA hit specificity evaluation. Because recent studies have suggested that siRNA specificity is not absolute and off-target effects can be induced by several different mechanisms (13–20), we tested whether our hits were specific. We used siRNA at the lowest possible effective concentration and correlated phenotypic outcome with siRNA-mediated knockdown using multiple siRNAs that target different regions of the same gene. To begin choosing the best hits out of our initial 23, we first assessed the magnitude of the individual siRNA-induced cell death phenotypes and determined whether similar effects using multiple siRNAs could be observed in several different phenotypic assays and cell lines. Four different siRNA duplexes for each of the 23 different targets were examined using an apoptosis readout (caspase-3 assay), a cell proliferation readout (ATPlite assay), and a cell death readout (Toxilight assay). In these assessments, we found that 10 out of 23 hits passed our initial criteria in that at least two siRNAs per target reproducibly induced significant cell death phenotypes in at least two human tumor cell lines using all assay formats. To determine siRNA specificity for these top 10 hits, we next examined the correlations between siRNA-mediated knockdown and cell death phenotypes

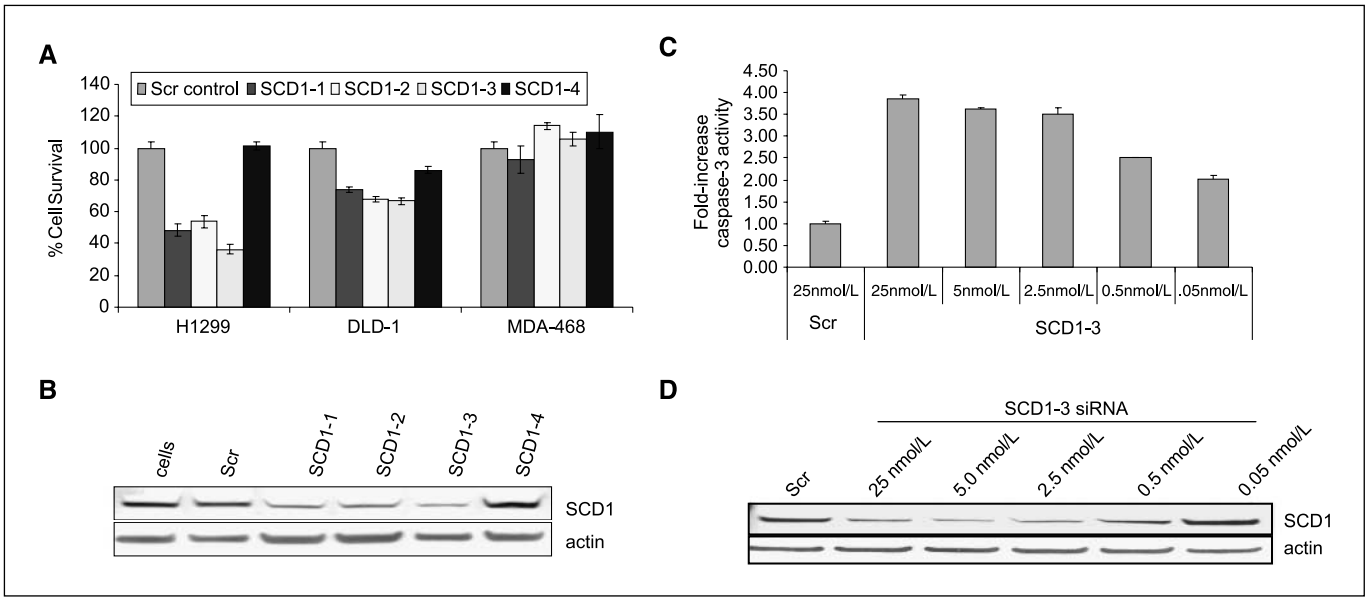

Figure 2. siRNAs against SCD1 induce a cell death phenotype that correlates with reduced protein levels. *A,* H1299, DLD-1, and MDA-468 cells were transfected with siRNAs (25 nmol/L) against SCD1 or with a universal negative scrambled control siRNA, and cell death was determined 72 h later using an ATPlite assay. *B,* H1299 cells were transfected with four different siRNAs against SCD1 using 25 nmol/L dose, and total protein expression levels were determined 72 h post-transfection using Western blot analysis. *C,* H1299 cells were transfected with SCD1-3 siRNA using a dose response (0.05–25 nmol/L siRNA), and cell death was measured 72 h later using a caspase-3 activation assay. *D,* SCD1 protein expression levels were determined from SCD1-3 siRNA-transfected H1299 cells by Western blot analysis 72 h post treatment.

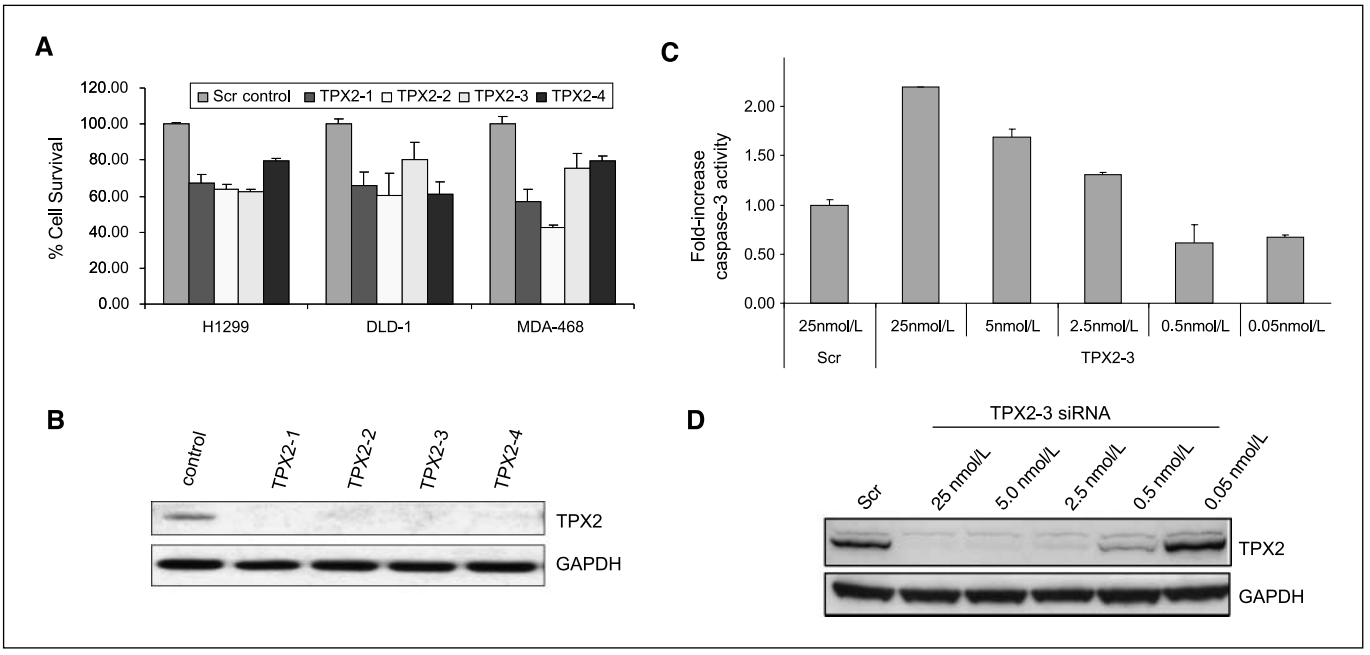

Figure 3. siRNAs against TPX2 induce a cell death phenotype that correlates with reduced target protein levels. *A,* H1299, DLD-1, and MDA-468 cells were transfected with siRNAs (25 nmol/L) against TPX2 or with a universal negative scrambled control siRNA, and cell death was determined 72 h later using an ATPlite assay. *B,* H1299 cells were transfected with four different siRNAs (25 nmol/L dose), and total protein expression levels were determined 48 h post-transfection using Western blot analysis. *C,* H1299 cells were transfected with TPX2-3 siRNA using a dose response (0.05–25 nmol/L siRNA), and cell death was measured 72 h later using a caspase-3 activation assay. *D,* TPX2 protein expression levels derived from TPX2-3 siRNA-transfected H1299 cells were determined at 48 h by Western blot analysis.

using siRNA doses ranging from 0.5 to 25 nmol/L and found that only 3 out of these top 10 siRNA hits showed good agreement between cellular phenotype and target knockdown.

One of these hits was SCD1, a well-defined enzyme that catalyzes the rate-limiting step in the biosynthesis of monounsaturated fatty acids that predominate in triglycerides, phospholipids, and cholesterol esters. Unbalanced levels of these fatty acids have been implicated in obesity, atherosclerosis, insulin sensitivity, and carcinogenesis (25, 26). As shown in Fig. 2*A* and *B*, several different siRNAs inhibited cell proliferation in H1299 and DLD-1 cells that correlated with SCD1 protein knockdown. This siRNA-mediated decrease in cell survival correlated with a 3-fold or greater increase in caspase-3 activity (data not shown). To further confirm that cell death is mediated by SCD1-specific siRNA, we tested siRNA potency by treating the H1299 cells with SCD1 siRNA-3 in a dose-response fashion (0.05–25 nmol/L) and then harvested these cells for cell death assays and Western blot analysis. These studies revealed that SCD1 siRNA is active at picomolar concentrations and can induce cell death as shown by caspase-3 activation in a dose-dependent manner (Fig. 2*C* and *D*).

In addition to SCD1, we identified siRNA hits specific to Ran and TPX2. Ran is a small GTPase that belongs to the RAS superfamily and plays a critical role in mediating the effects of several mitotic factors that include TPX2 (27). TPX2 promotes microtubule assembly around chromosomes by targeting Aurora A kinase to the spindle microtubules (28). Aurora A plays an important role in centrosome maturation and in mitotic spindle assembly and serves as a key regulator of genome stability (29). As shown in Figs. 3*A* and 4*A*, all four siRNAs directed against either TPX2 or Ran inhibited cell proliferation in multiple human tumor cell lines. Importantly, the observed siRNA-induced cell death phenotypes resulted in the reduction of the respective target protein levels (Figs. 3*B* and 4*B*). Multiple siRNAs against Ran and TPX2 also induced caspase-3 activation by at least 2-fold and decreased colony formation by ~50% in soft agar (data not shown). To further validate the specificities of these siRNAs, H1299 cells were treated with TPX2 siRNA-3 or Ran siRNA-1 over a range of concentrations and then harvested for cell death assays (caspase-3 and Toxilight assays) and Western blot analysis. Both TPX2 and Ran siRNAs induced cell death phenotypes in a dose-dependent manner, and this correlated well with siRNA-mediated knockdown of TPX2 or Ran (Figs. 3*C* and *D* and 4*C* and *D*). Ran siRNA is active at picomolar concentrations, whereas TPX2 siRNA is active at low nanomolar concentrations.

TPX2 and Ran are required for cell survival in K-Ras–activated cells. Because many of the human tumor cell lines examined in our validation studies contain Ras mutations, we next investigated whether our hits may cooperate with the Ras oncogene for cell cycle progression and tumor cell growth. Given the fact that Ras-activating mutations are one of the more prevalent genetic changes in cancer (30), data to suggest SCD1, Ran, or TPX2 as required for cell survival in mutant Ras activated cells would strengthen these as important anticancer drug targets. To address this question, we evaluated a pair of human isogenic cancer cell lines that contain an activated *K-Ras* allele (DLD-1 parental), or a wild-type *K-Ras* allele (DKS-8 isogenic counterpart; ref. 31). Characterization of these lines revealed that they have similar growth rates as measured by a cell proliferation assay (data not shown), consistent with previously published observations (31). Using two different cell death assay formats, we observed that multiple Ran and TPX2, but not SCD1, siRNAs induced differential cell killing in the DLD-1 cells expressing a mutant activated K-Ras versus the control DKS-8 cells expressing wild-type K-Ras (Fig. 5*A*), although all siRNAs induced equivalent target knockdown in the

DLD-1 and DKS-8 cell lines (data not shown). These phenotypes also correlated with an observed 20% to 30% differential in percent growth inhibition (Fig. 5*B*). Transfection with a positive Plk1 siRNA resulted in similar cell death phenotypes in both lines (data not shown). Increased cell death was also induced by multiple siRNAs transfected into an additional isogenic cell line that contained only the activated mutant *K-Ras* allele (DKO-1), as compared with isogenic clones containing only the wild-type *K-Ras* allele (DKO-3, DKO-4; Fig. 5*C*), suggesting that TPX2 and Ran may play a role in the K-Ras–dependent survival pathway.

Ran and TPX2 knockdown in activated mutant K-Ras cells induces differential cell death that is preceded by either transient G_2-M arrest or S-phase arrest phenotypes. We next investigated the possible biological mechanisms for the toxicity we observed with Ran and TPX2 siRNAs in the context of a mutant activated K-Ras background. Given the role these targets play in regulating cell cycle progression (27), we hypothesized that knockdown of Ran and/or TPX2 in the context of activated K-Ras may exaggerate cell cycle aberrations that trigger apoptotic cell death. We examined siRNA effects on DLD-1 and DKS-8 cell cycle progression and DNA synthesis by measuring the incorporation of BrdUrd-FITC and propidium iodide label into DNA using flow-cytometric analysis. Although the distribution of logarithmically growing cells in G_1, S, and G_2-M was similar for scrambled siRNA-treated DLD-1 and DKS-8 cells, we observed marked differences in cell cycle distribution among the isogenic pair treated with TPX2 and Ran siRNAs (Fig. 6). Specifically, we observed that treatment of DLD-1 cells with TPX2 siRNAs resulted in cells transiently accumulating in G_2-M accompanied by ~10% polyploid (8N) population as early as 24 h. This siRNA-induced accumulation in G_2-M is consistent with previous studies showing that siRNA-mediated knockdown of TPX2 results in a mitotic arrest that is associated with abnormal spindle formation (32, 33). By 48 h, however, TPX2 siRNA treatment of the K-Ras–activated cells resulted in the eventual bypass of this G_2-M arrest as shown by the reentry of cells into the G_1 phase (Fig. 6). This observed reentry and, therefore, recycling of K-Ras–activated cells in the absence of functional TPX2 were associated with enhanced apoptotic cell death by 72 h (Fig. 5*A* and *B*). In contrast, treatment of K-Ras wild-type DKS-8 cells with TPX2 siRNA resulted in a stable G_2-M arrest by 48 h (Fig. 6), followed by a lesser cell death phenotype at 72 h (Fig. 5*A* and *B*). By lengthening the time course study of cell death up to 6 days, the TPX2 siRNA-transfected DKS-8 cells maintained the stable G_2-M arrest and lesser cell death phenotypes (data not shown), suggesting that the DLD-1 cells do not simply reach the stage of cell death more quickly, but rather, the cell death differential is due to preferential sensitivity to TPX2 depletion. Marked differences were also seen in cell cycle distribution between the isogenic K-Ras pair after treatment with Ran siRNA. In contrast to the DKS-8 siRNA-treated cells, Ran siRNA-treated DLD-1 cells expressing mutant activated K-Ras resulted in ~2-fold increase in the S-phase population by 48 h as marked by a dramatic decrease in BrdUrd incorporation (Fig. 6). This S-phase arrest was also associated with a concomitant increase in apoptotic cells by 72 h compared with the siRNA-treated DKS-8 cells (Fig. 5*A* and *B*). Figure 6*B* and *C* reveals representative quantitations of cell cycle populations 24 and 48 h posttreatment of H1299 cells with scrambled, TPX2, or Ran siRNAs.

Discussion

With the advent of large-scale gene knockdown using siRNA libraries, it has become possible to systematically screen for genes that are linked to tumorigenesis and to identify novel drug targets. Indeed, in this study, we implemented an RNAi-based screen that resulted in the successful identification of SCD1, TPX2, and Ran as

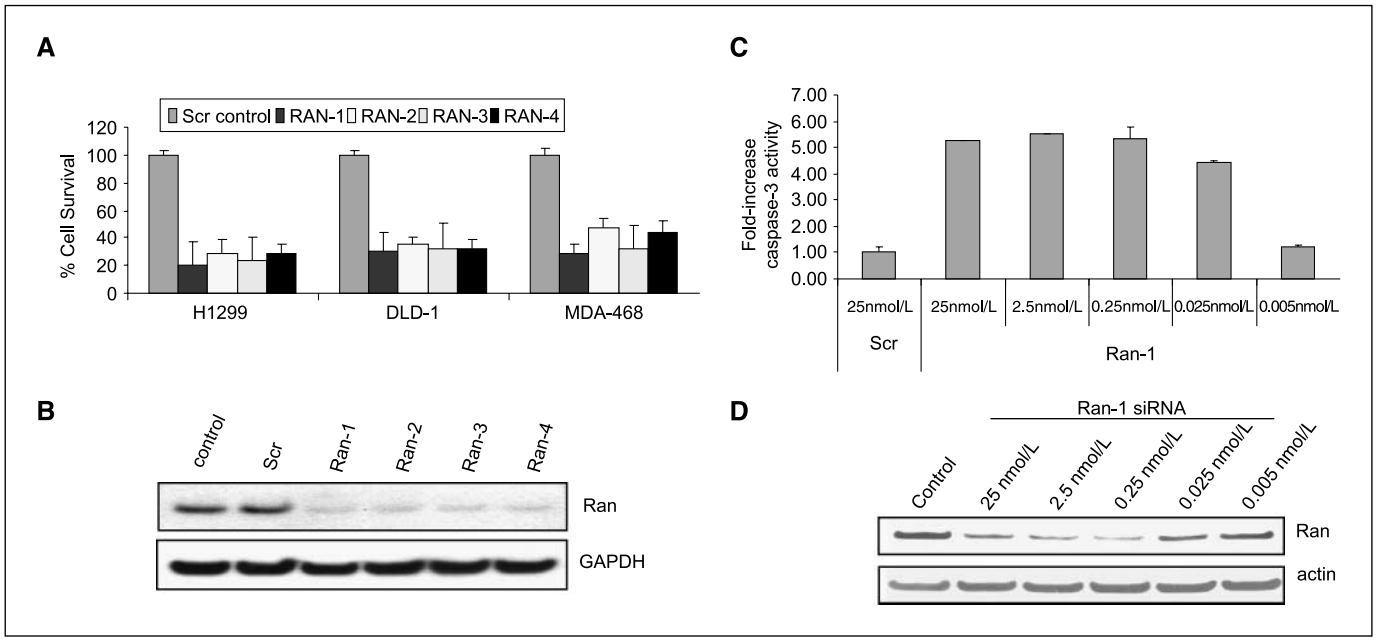

Figure 4. siRNAs against Ran induce a cell death phenotype that correlates with reduced target protein levels. *A,* H1299, DLD-1, and MDA-468 cells were transfected with siRNAs (25 nmol/L) against Ran or with a universal negative scrambled control siRNA, and cell death was determined 72 h later using an ATPlite assay. *B,* H1299 cells were transfected with four different siRNAs (25 nmol/L dose), and total protein expression levels were determined 48 h post-transfection using Western blot analysis. *C,* H1299 cells were transfected with Ran-1 siRNA using a dose response (0.005–25 nmol/L siRNA), and cell death was measured 72 h later using a caspase-3 activation assay. *D,* Ran protein expression levels derived from Ran-1 siRNA-transfected H1299 cells were determined at 72 h by Western blot analysis.

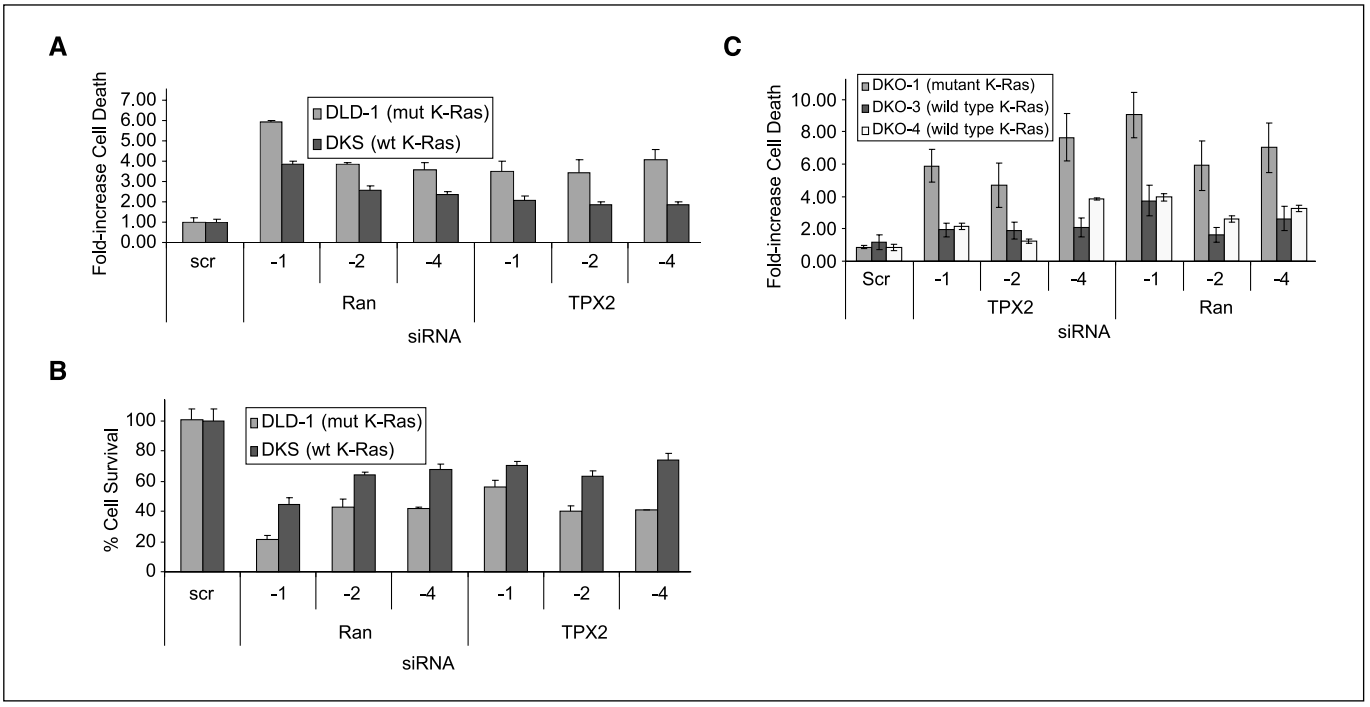

Figure 5. Screening of Ran and TPX2 siRNAs in K-Ras isogenic clones reveals K-Ras dependence on Ran and TPX2 for cell survival. *A* and *B,* Ran and TPX2 siRNAs were transfected into the K-Ras mutant and K-Ras wild-type isogenic cell lines at 25 nmol/L and assayed 72 h post-transfection by the Toxilight and ATPlite assays. *C,* K-Ras disrupted (*DKO-1*) and K-Ras wild-type (*DKO-3, DKO-4*) clones were transfected with Ran and TPX2 siRNAs (5 or 25 nmol/L, respectively). Cell death was measured at 72 h post-transfection using the Toxilight assay.

playing a role in human tumor cell survival. In addition to these targets, our existing library undoubtedly contains siRNAs against multiple other genes essential to tumor cell growth. These additional hits may have been missed due to the context in which the screen was done and our selection criteria, namely, screening the library using a specific cell death assay and a single time point and assuming that the majority of siRNAs reliably knocked down the targeted protein at a sufficient level to induce cell death. Furthermore, one of the main complications arising from RNAi-based screens is the lack of siRNA specificity. We have shown in this study, however, that potentially important targets can be identified, providing that follow-up experiments are conducted to check for siRNA specificity.

SCD1 is an enzyme found in the endoplasmic reticulum that is involved in the synthesis of unsaturated fatty acids. SCD1 acts in conjunction with two accessory proteins, flavoprotein cytochrome b_5 reductase and the electron acceptor cytochrome b_5, to catalyze the incorporation of a single double bond between carbons 9 and 10 of saturated fatty acyl-CoA substrates, producing monounsaturated fatty acids (25). The predominant substrates for SCD1 are palmitoyl- and stearoyl-CoA (25). It is well known that monounsaturated fatty acids can serve as mediators of signal transduction and cellular differentiation, and unbalanced levels of these mediators have been implicated in obesity, atherosclerosis, insulin sensitivity, and carcinogenesis (26). In the present study, we have shown that knockdown of SCD1 protein by siRNA induces a potent cell death phenotype *in vitro*, suggesting that endogenous synthesis of monounsaturated fatty acids may indeed be essential for tumor cell growth, although further *in vivo* validation studies are needed. However, validation for SCD1 as an anticancer target also comes from recent literature evidence correlating enhanced SCD1 enzymatic activity with tumorigenesis (34, 35) and citing SCD1 overexpression in a variety of carcinomas (34, 36–38). Studies have well documented that stearic acid and conjugated linoleic acid have strong inhibitory effects on mammary carcinogenesis both *in vitro* and *in vivo*, which has been suggested to be due, in part, to the ability of conjugated linoleic acid to reduce monounsaturated fatty acids through the reduction of SCD1 activity (39). Saturated free fatty acids have been shown to inhibit tumor cell growth and promote apoptosis, whereas unsaturated free fatty acids can block these effects. In other studies, stable knockdown of human SCD1 using antisense cDNA-transfected SV40-transformed human fibroblasts resulted in high levels of triacylglycerol and saturated free fatty acids. This profile correlated with a slower growth rate, loss of anchorage-independent cell growth, and enhanced apoptosis (35). Based on these observations, it has been proposed that an increased ratio of monounsaturated fatty acids to saturated fatty acids, and hence, a more fluid membrane environment, acts as a primary trigger of active cell division. Indeed, the lipid products of SCD1 are used for modulating cell membrane structure and energy metabolism (25). SCD1 expression is known to be up-regulated by growth factors such as platelet-derived growth factor, fibroblast growth factors FGF2 and FGF4, and transforming growth factor-β through a Smad signaling pathway (40).

The other two validated hits, Ran and TPX2, may also serve as valuable anticancer targets because they both have an integral link to the Aurora A activation pathway that has been well documented to be critical in cell cycle progression and tumorigenesis (29). Support for targeting the Aurora A kinase pathway comes from the proven *in vivo* efficacy of small-molecule Aurora kinase inhibitors using human tumor xenograft models (29, 41). Ran is a small GTPase that belongs to the RAS superfamily that has been found to be occasionally amplified in common tumors and plays a critical role in the translocation of macromolecules through the nuclear

pore complex, regulating cell cycle progression and DNA synthesis (42), suggesting that small molecules targeting Ran *in vivo* may be efficacious, but further studies are required. Although it may be difficult to target the Ran GTPase using small-molecule–based therapies, it may be possible as suggested by the description of small-molecule inhibitors against a Rac GTPase shown to inhibit human tumor cell growth *in vitro* (43).

The GTP-bound form of Ran (Ran-GTP) also coordinates spindle microtubule assembly around the chromosomes during mitosis (44, 45). Nuclear localization of Ran-GTP requires interaction with regulator of chromosome condensation 1, which is the guanine nucleotide exchange factor for Ran. In our study, we have shown that down-regulation of Ran in a variety of human tumor cell lines results in apoptotic cell death. Given one of its roles in DNA synthesis, one would expect Ran inactivation to induce aberrations in the cell cycle that precede apoptosis. Indeed, by expanding our analysis to include more relevant cell-based models such as the K-Ras isogenic cell line pair, we found that specific treatment with Ran siRNA differentially reduced the survival of activated K-Ras–transformed cells (DLD-1) that was associated with an increase in S-phase arrest compared with the normal isogenic counterpart, in which the mutant K-Ras gene had been disrupted (DKS-8). Ran may cooperate with activated K-Ras to drive tumor cell cycle progression, and disruption of this synergy results in irreversible S-phase arrest and eventual cell death.

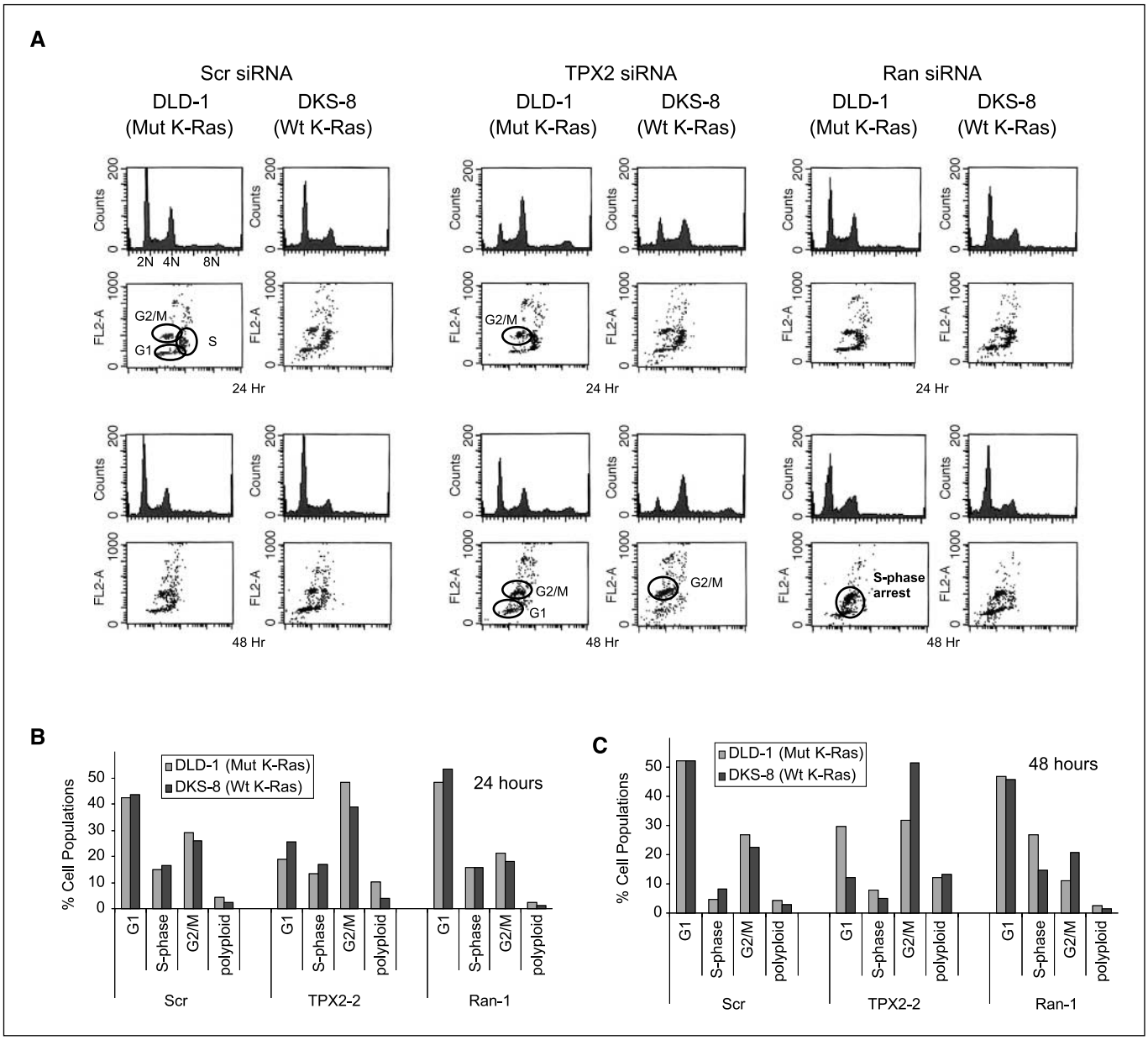

Figure 6. TPX2 siRNA-induces a transient G_2-M arrest, and Ran siRNA induces an S-phase arrest in the DLD-1 cells. *A,* flow-cytometric dot plots display simultaneous analysis of S-phase DNA synthesis (*x*-axis), as determined by a 1-h BrdUrd pulse labeling of cells at 72 h after TPX2-2 siRNA (25 nmol/L), Ran-1 siRNA (25 nmol/L), or universal scrambled siRNA (25 nmol/L) treatment and total DNA content (*y*-axis), as determined by propidium iodide staining. The corresponding histogram plots display analysis of total DNA content as determined by propidium iodide staining. Cell cycle populations are characterized as G_1 (2 N DNA content), S-phase (BrdUrd incorporation), G_2-M (4 N DNA content), and polyploidy (8 N DNA content or greater). *B* and *C,* representative quantitations of percent cell cycle populations 24 and 48 h post treatment of H1299 cell line with scrambled, TPX2, or Ran siRNAs.

Ran may thus serve as an anticancer target in tumors expressing the activated K-Ras oncogene, enabling one to better define selective tumor cell cytotoxicities.

TPX2 is a microtubule-associated protein that serves as one of the essential downstream targets of Ran-GTP during cell division and is found to be frequently amplified in common tumors (27, 46). One recent study identified TPX2 as a potential candidate oncogene targeted for amplification in both pancreatic and non–small cell lung cancers based on high-resolution comparative genomic hybridization analysis (47). This amplification seemed to correlate with overexpression of genes involved in spindle formation and mitotic progression such as Bub1 and the Aurora kinases. In terms of function, TPX2 is normally bound to a nuclear transport receptor, importin β, via the adaptor protein importin α that regulates TPX2 import into the nucleus at interphase. The TPX2-importin complex is dissociated by the binding of Ran-GTP to importin β, thereby releasing TPX2 to promote microtubule assembly around chromosomes (27, 46). Indeed, TPX2 has been shown to play roles in spindle formation using *Xenopus* egg extracts and in normal spindle morphology and centrosome integrity during mitosis using RNAi (32, 33). Interestingly, one of the main functions of TPX2 is the targeting of the Aurora A kinase to the spindle microtubules. TPX2-Aurora A interaction leads to the activation of Aurora A kinase and, in turn, the phosphorylation of TPX2 by this kinase (28, 48). Aurora A plays an important role in centrosome maturation and in mitotic spindle assembly, serves as a key regulator of genome stability, and is frequently amplified in clinically aggressive tumors (29). TPX2 may thus serve as a valuable anticancer target *in vivo* given its direct link in the Aurora A activation pathway that has been shown to be critical in tumor progression as shown by the *in vivo* efficacy of several different Aurora kinase inhibitors (29, 41). Provided that TPX2 is amenable to small-molecule inhibitors, targeting TPX2 over Aurora A may have its advantages given that blocking TPX2 binding to and, thus, activation of Aurora A kinase provides a higher specificity that may not be achievable with conventional kinase inhibitors. Nonetheless, small molecules or siRNAs targeting TPX2 have not been reported, and it may be difficult to obtain such agents that are therapeutically useful.

In the present study, we have shown that down-regulation of TPX2 in a variety of human tumor cell lines results in apoptotic cell death, and that this phenotype seems to be preferentially enhanced in activated K-Ras–transformed cells, further strengthening TPX2 as an ideal anticancer target. Mechanistic studies through cell cycle analysis of a K-Ras isogenic cell line pair revealed that TPX2 knockdown resulted in gross cell cycle aberrations as shown by a transient G_2-M arrest and a small percentage of polyploidy, followed by reentry into the cell cycle and a concomitant increase in apoptotic cell death compared with the siRNA-treated wild-type K-Ras cells. Given that one of the Ras downstream pathways, Raf-1/mitogen-activated protein (MAP)/extracellular signal-regulated kinase kinase/MAP kinase (MAPK), has been implicated to play a role in driving cells through the G_2-M phase (49, 50), coupling TPX2 siRNA-induced spindle formation abnormalities with activated K-Ras–mediated G_2-M progression would be consistent with the potentiation of cell death.

In summary, our present findings highlight the utility of using unbiased RNAi-based screens to identify essential survival proteins that may serve as potentially important targets for cancer therapy. By expanding our analysis to K-Ras isogenic cell line pairs, we also show a possible codependence between Ran or TPX2 and K-Ras expression for cell survival.

Acknowledgments

Received 11/8/2006; revised 1/18/2007; accepted 2/19/2007.

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We thank Jeremy Packer and Randy Metzger for the preparation of the gene list that represents the siRNA library. We also thank Paul Kroeger and Xiaoan Ruan for performing the QPCR experiments that aided in the siRNA-mediated mRNA knockdown/phenotype correlation studies and Randy Bell and George Sheppard for critical evaluation of the manuscript.

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