

# Topoisomerase levels determine chemotherapy response *in vitro* and *in vivo*

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Topoisomerase poisons are chemotherapeutic agents that are used extensively for treating human malignancies. These drugs can be highly effective, yet tumors are frequently refractory to treatment or become resistant upon tumor relapse. Using a pool-based RNAi screening approach and a well characterized mouse model of lymphoma, we explored the genetic basis for heterogeneous responses to topoisomerase poisons *in vitro* and *in vivo*. These experiments identified *Top2A* expression levels as major determinants of response to the topoisomerase 2 poison doxorubicin and showed that suppression of *Top2A* produces resistance to doxorubicin *in vitro* and *in vivo*. Analogously, using a targeted RNAi approach, we demonstrated that suppression of *Top1* produces resistance to the topoisomerase 1 poison camptothecin yet hypersensitizes cancer cells to doxorubicin. Importantly, lymphomas relapsing after treatment display spontaneous changes in topoisomerase levels as predicted by *in vitro* gene knockdown studies. These results highlight the utility of pooled shRNA screens for identifying genetic determinants of chemotherapy response and suggest strategies for improving the effectiveness of topoisomerase poisons in the clinic.

Chk2 | doxorubicin | RNAi screen | Top1 | Top2A

A myriad of genetic factors influence the efficacy of cancer chemotherapy, including both somatic changes in the tumor itself as well as genetic polymorphisms present in the patient. These factors include increased expression of detoxification pumps that prevent access of the drug to its target (1), point mutations that disrupt the drug–target interaction (2, 3), and mutations in stress response pathways [e.g., *p53* loss (4)]. To tailor treatment successfully to the individual patient, a more complete understanding of the genetic determinants of therapy response is necessary.

RNA interference (RNAi) exploits a mechanism of gene regulation whereby double-stranded RNAs are processed by a conserved cellular machinery to suppress the expression of genes containing homologous sequences (5). Importantly, libraries of DNA-based vectors encoding short hairpin RNAs (shRNAs) capable of targeting most genes in the human and mouse genomes have been produced and enable forward genetic screens to be performed in mammalian cells. Indeed, by using human tumor-derived cell lines treated *in vitro*, RNAi has been used to evaluate potential drug targets (6) or to investigate mechanisms of drug action and drug resistance by screening for new molecules that modulate the response of tumor-derived cell lines to a given chemotherapeutic agent (7–10).

Here, we evaluate the suitability of combining mouse models and RNAi to identify genetic modifiers of drug action in tumors in their natural site. Initially, we chose to investigate resistance to doxorubicin in the *Eμ-Myc* mouse lymphoma system. Doxorubicin (Adriamycin) is an anthracycline DNA-damaging agent that exerts its effects primarily by targeting of the topoisomerase 2 activity and DNA intercalation (11). Along with etoposide and the camptothecin derivatives, doxorubicin is one of several topoisomerase-targeted drugs currently used as front-line ther-

apies for a wide variety of cancers. The *Eμ-Myc* lymphoma system has been a highly tractable model for studying the genetic determinants of chemotherapeutic response *in vivo* in an immunocompetent setting (12), and recently we have adapted RNAi-based loss-of-function technology for use this model (13–15). Here, we demonstrate that the *Eμ-Myc* system can successfully identify crucial mediators of the response to topoisomerase poisons. These genes validate for relevance *in vivo*, suggesting strategies for improved clinical use of these drugs.

## Results

### RNAi Screens Identify shRNAs Mediating Doxorubicin Resistance.

Because *in vivo* studies of drug sensitivity and resistance require stable gene knockdown, we performed our initial *in vitro* screens using retrovirally encoded shRNAs based on the MiR-30 microRNA (16). Importantly, these shRNAs can stably and efficiently knockdown target genes when expressed at single copy in the genome (13). We chose to survey shRNAs targeting the “cancer 1000,” a set of known or putative cancer-relevant genes compiled by manual curation, microarray expression data, and literature mining (17). To improve gene knockdown and facilitate *in vivo* experiments (13), all of the existing murine shRNAs targeting the cancer 1000 set ( $\approx 2,300$  shRNAs, two to three shRNAs per gene) were cloned into a murine stem cell virus (MSCV)-based vector that coexpressed green fluorescent protein.

Our initial screens for shRNAs capable of conferring doxorubicin resistance used *p19<sup>ARF</sup>-/-*; *Eμ-Myc* lymphoma cells, which retain the *p53* tumor suppressor and an intact DNA damage response (18, 19). shRNA pools were introduced into lymphoma cells by retroviral transduction, and infected cultures were treated with doxorubicin at doses that typically would kill 70–95% of cells in 24 h.

Three independent approaches were used to identify shRNAs enriched after doxorubicin treatment [supporting information (SI) Fig. S1]. Specifically, the library was screened by using either (i) single treatments of lymphoma cells transduced with low-complexity shRNA pools or, alternatively, (ii) single or (iii) serial treatments of lymphoma cells transduced with the whole shRNA set. Standard DNA sequencing of amplified provirus shRNAs was used to identify constituent shRNAs and to determine their relative representation in the treated and untreated cell populations (Fig. S2). Similar results were also produced by using high-throughput shRNA deconvolution via DNA microarrays

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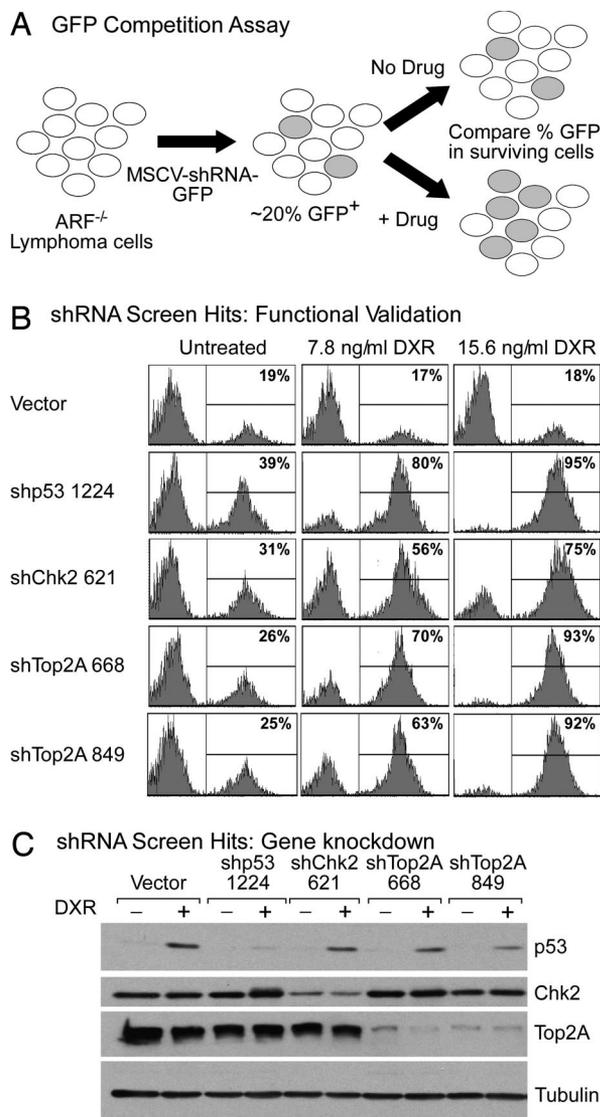
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**Fig. 1.** A rapid RNAi enrichment screen identifies mediators of doxorubicin resistance. (A) The GFP competition assay. Differential survival of shRNA-transduced cells (green) relative to control cells (colorless) is assayed by changes in the percentage GFP in the surviving cell population. (B) GFP competition assay data from lymphoma cells infected with the indicated shRNA either untreated or 24 h after doxorubicin (DXR) treatment at the indicated doses. (C) Immunoblotting of lysates from lymphoma cells transduced with shRNAs targeting *p53*, *Chk2*, and *Top2A* either untreated or treated for 8 h with 31 ng/ml doxorubicin to stabilize *p53*. Tubulin serves as a loading control.

and Solexa deep sequencing, illustrating the potential for pooled screens of expanded scope (D.L.B., S.W.L., J. Zuber, and E. Hodges, unpublished work). Irrespective of the screening approach, shRNAs targeting *p53*, *Chk2*, and *Top2A* (two independent shRNAs) were repeatedly identified as being enriched upon doxorubicin treatment. Additional shRNAs were also identified as becoming enriched after drug treatment via one strategy or another (Fig. S2), and these should be the subject of future studies.

To validate the screening results, we retested the major shRNA hits in an “*in vitro* competition assay.” This assay examines the impact of specific shRNAs on therapy response in partially transduced cell populations, using GFP-based flow cytometry to track the survival advantage or disadvantage

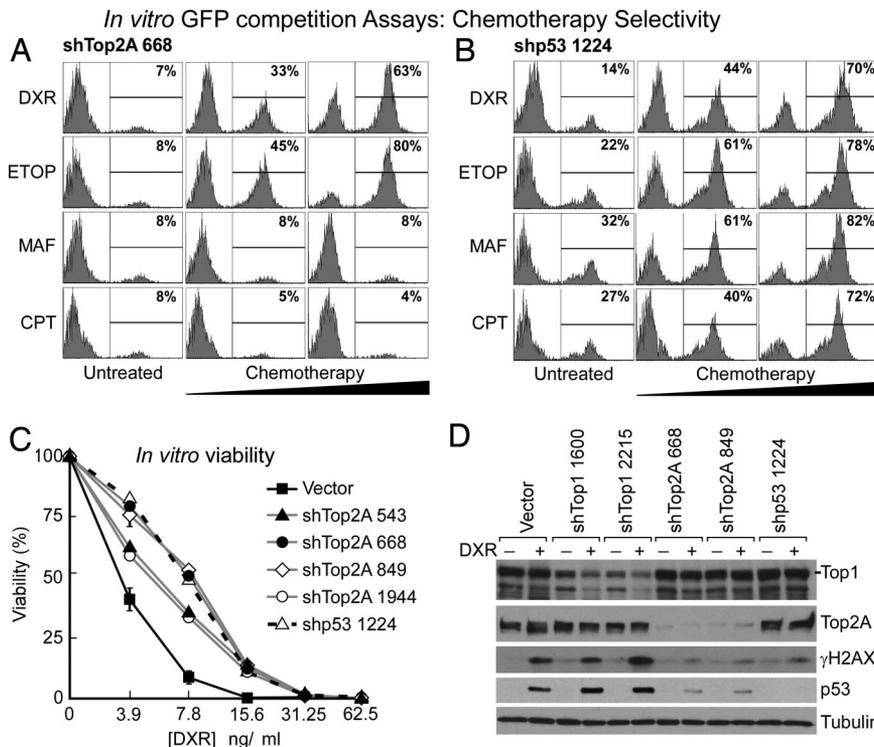
conferred by specific shRNAs (Fig. 1A). shRNAs targeting *p53*, *Chk2*, and *Top2A* successfully validated in the competition assay: the shRNAs were dramatically enriched in cell populations within 24 h after doxorubicin treatment (Fig. 1B). Additionally, these shRNAs effectively suppressed expression of their intended target (Fig. 1C).

*p53* and *Chk2* are key components of DNA damage response pathways and, indeed, *p53* loss confers resistance to doxorubicin in the *E $\mu$ -Myc* transgenic model (13, 20). Importantly, multiple shRNAs targeting *Chk2* promoted doxorubicin resistance, suggesting that the effects of these shRNAs were “on target,” i.e., specifically due to *Chk2* gene knockdown (Fig. 1B and Fig. S3). Although *Chk2* can sensitize cells to DNA-damaging agents in some contexts (21, 22), our results are consistent with a role for *Chk2* in signaling *p53*-dependent apoptosis in lymphoid cells (20, 23). These results suggest we can identify relevant mediators of drug resistance using pool-based RNAi screening approaches.

**Top2A shRNAs Cause Resistance Specifically to Topoisomerase 2 Poisons.** shRNAs targeting Topoisomerase 2 $\alpha$  (*Top2A*) were the most frequently recovered shRNAs from doxorubicin-treated cells, with at least two independent shRNAs isolated per screen. *Top2A* is a target of the drug doxorubicin (11) and is an essential gene in mammals (24). Unlike typical enzyme inhibitors where knockdown of the drug target would be expected to mimic drug action and promote cell death, doxorubicin is a topoisomerase poison that stabilizes the cleavable complex consisting of double-stranded DNA breaks to which the enzyme is covalently attached. Doxorubicin therefore causes excessive double-stranded DNA breaks via unresolved cleavable complexes in a topoisomerase-dependent manner, thereby explaining how *Top2A* down-regulation might confer doxorubicin resistance (25). Remarkably, even very potent knockdown of *Top2A* (Fig. 1C) had little, if any, impact on cell proliferation in the absence of drug treatment, suggesting that normal cell proliferation can proceed with relatively low *Top2A* expression (data not shown).

Although previous work has suggested a relationship between *Top2A* levels and doxorubicin sensitivity (26), the effect has not been studied extensively or validated *in vivo*. The effects of *Top2A* knockdown were specific to topoisomerase 2 poisons: shTop2A caused resistance to another, structurally unrelated TOP2A poison, etoposide, but not to the alkylating agent maphosphamide (an active metabolite of cyclophosphamide) nor the topoisomerase 1 poison camptothecin (Fig. 2A). In contrast, an shRNA targeting *p53* caused cross-resistance to these different agents (Fig. 2B). The drug response-modifying effects of *Top2A* knockdown were likely “on target”: four of four *Top2A* shRNAs mediated resistance specifically to topoisomerase 2 poisons (Fig. 2C and Fig. S4A and C). As expected, cells with reduced TOP2A levels displayed a diminished DNA damage signal and response, as shown by lower  $\gamma$ -H2AX signal, less *p53* stabilization, and less apoptosis upon doxorubicin treatment (Fig. 2D and Fig. S5). Accordingly, the ability of *Top2A* shRNAs to promote doxorubicin resistance was attenuated in *p53*-null *E $\mu$ -Myc* lymphoma cells (Fig. S4B), although clearly some signals downstream of chemotherapy-induced DNA damage are *p53*-independent (27).

**Top2A shRNAs Confer Resistance to Doxorubicin *in Vivo*.** To test the role of *Top2A* in doxorubicin resistance *in vivo*, *E $\mu$ -Myc;Arf<sup>-/-</sup>* lymphoma cells were infected *in vitro* with shTop2A or a control vector and transplanted via tail vein injection into multiple syngeneic recipient mice. Tumor-bearing recipient mice were then treated with the maximum tolerated dose of doxorubicin (Fig. S6). *Top2A* knockdown caused doxorubicin resistance *in vivo* as measured by an *in vivo* competition assay (an increase in the percentage of GFP-positive cells after drug treatment; Fig. 3A) and reduced tumor-free (Fig. 3B) and overall survival (data



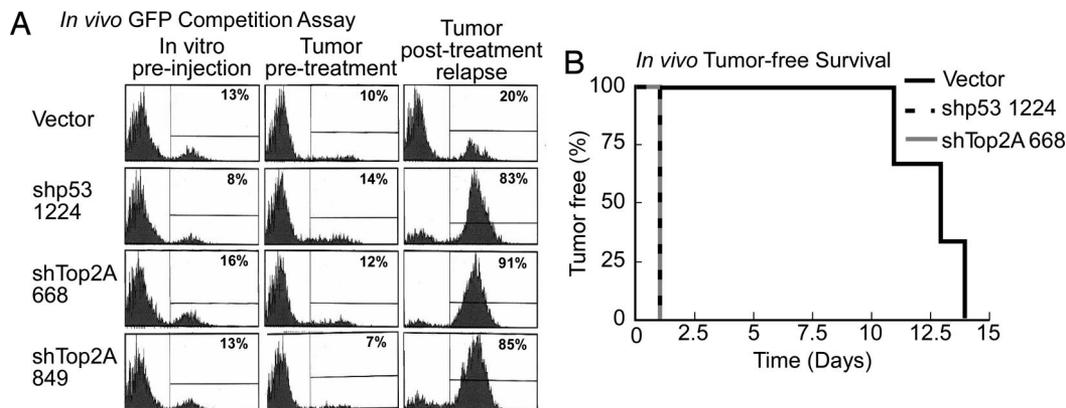
**Fig. 2.** Suppression of *Top2A* expression causes resistance to topoisomerase 2 poisons *in vitro*. (A and B) Flow cytometric analyses of lymphoma cells expressing shTop2A 668 (A) or shp53 1224 (B) after 24 h of the indicated drug treatments. DXR, doxorubicin; ETOP, etoposide; MAF, maphosphamide; CPT, camptothecin. (C) Lymphoma cells, transduced singly with four independent *Top2A* shRNAs, were puromycin-selected and treated with doxorubicin for 24 h at the indicated doses. Viability was assayed by flow cytometry (FSC versus SSC) and plotted relative to untreated controls. Error bars are  $\pm$ SEM from three replicates. (D) Immunoblotting of lymphoma cell lysates expressing no short hairpin (Vector) or *Top1*, *Top2A*, or *p53* shRNAs in the presence or absence of doxorubicin (DXR; 15.6 ng/ml for 8 h).

not shown). These results demonstrate that reduced *Top2A* expression is a bona fide mechanism of drug resistance *in vivo*.

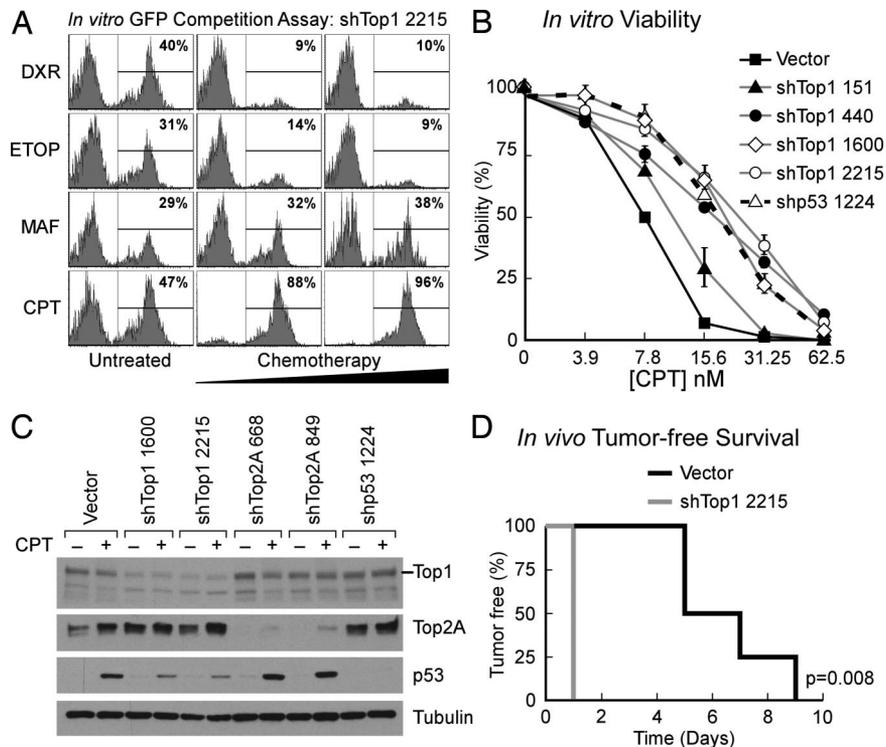
**Top1 shRNAs Confer Resistance to Topoisomerase 1 Poisons *in Vitro* and *in Vivo*.** *TOP2A* is not the only topoisomerase targeted by front-line anticancer therapeutics. Topoisomerase 1 (TOP1) is the target of camptothecin (28, 29) and its derivatives irinotecan (Camptosar/CPT-11) and topotecan (Hycamtin). *TOP1*-deficient yeast are viable and resistant to camptothecin (30), but complete knockout of *Top1*, like *Top2A*, is lethal in mammals

(31). Prompted by our studies on doxorubicin and *Top2A*, we tested whether *Top1* knockdown could induce camptothecin resistance in cancer cells. Indeed, *Top1* knockdown in *E $\mu$ -Myc;Arf<sup>-/-</sup>* lymphomas caused resistance specifically to camptothecin (Fig. 4A), and the effects were reproducible by using multiple independent *Top1* shRNAs (Fig. 4B, Fig. S7, and Fig. S8). Even modest *Top1* knockdown achieved this cytoprotective effect (Fig. 4C). Importantly, this effect was also seen in human cells expressing a *TOP1* shRNA (Fig. S8C).

*p53* induction was compromised in shTop1-expressing lym-



**Fig. 3.** *Top2A* knockdown causes doxorubicin resistance *in vivo*. *In vivo* competition assay is shown. (A) GFP flow cytometry plots. Lymphoma cells were infected *in vitro* with GFP-tagged shTop2A 668 or 849, shp53, or vector control constructs (A Left). These cells were injected into the tail vein of syngeneic recipient mice (five mice per cohort) and were monitored daily for tumors by palpation. Upon tumor onset (day 0), one mouse from each cohort was killed, and lymphoma cells were assayed for percentage GFP<sup>+</sup> (A Middle). The remaining mice were treated with doxorubicin (10 mg/kg i.p. injection), and tumors were harvested upon relapse and assayed for percentage GFP<sup>+</sup> (A Right). (B) Kaplan–Meier tumor-free survival curves. Vector, shTop2A, and shp53 tumors were FACS-sorted to 100% GFP<sup>+</sup> before injection into recipient mice and DXR-treated as for A at day 0.



**Fig. 4.** *Top1* knockdown causes camptothecin resistance *in vitro* and *in vivo*. (A) *Top1* knockdown causes resistance to camptothecin but hypersensitizes to the topoisomerase 2 poisons, doxorubicin and etoposide, as shown by a GFP competition assay 24 h after drug treatment. (B) *In vitro* viability assays of puromycin-selected (shRNA-containing) cells for four independent shRNAs targeting *Top1*, after 24-h camptothecin treatment. Error bars are  $\pm$ SEM from three replicates. (C) Immunoblotting of *E $\mu$ -Myc;Arf<sup>-/-</sup>* lymphoma cell lysates with or without camptothecin (31 nM CPT, 8 h). (D) Kaplan–Meier survival curve. *E $\mu$ -Myc;Arf<sup>-/-</sup>* lymphomas were infected *in vitro* with vector control or shTop1 2215 and were FACS-sorted to 100% GFP<sup>+</sup> before injection into recipient mice. Upon lymphoma onset (day 0) mice were treated with irinotecan (CPT-11), a clinically relevant camptothecin derivative (50 mg/kg intraperitoneal injection daily for 2 days) and monitored for survival.

phoma cells treated with camptothecin, suggesting that these cells mounted a weaker DNA damage response (Fig. 4C). Accordingly, resistance was also attenuated in an *E $\mu$ -Myc;p53<sup>-/-</sup>* background (Fig. S7B). Mice harboring shTop1-expressing lymphomas displayed a reduced tumor-free survival compared with controls after treatment with irinotecan, indicating that reduced *Top1* expression promotes resistance to topoisomerase 1 poisons *in vivo* (Fig. 4D). Therefore, sufficient expression of *Top2A* or *Top1* is required to achieve a potent response to chemotherapeutic agents targeting each particular topoisomerase.

**Top1 shRNAs Enhance Sensitivity to Topoisomerase 2 Poisons.** The drug resistance phenotypes conferred by *Top1* shRNAs were specific for topoisomerase 1 poisons. For example, *Top1* knockdown had little effect on tumor cell sensitivity to the alkylating agent maphosphamide (Fig. 4A). Unexpectedly, *Top1* knockdown hypersensitized cells to the topoisomerase 2 poisons doxorubicin and etoposide (Fig. 4A), an effect reproduced with nine independent *Top1* shRNAs (Fig. S7 and Fig. S8). Furthermore, mice harboring transplanted lymphomas expressing *Top1* shRNAs showed an improved tumor-free survival compared with controls after irinotecan treatment (Fig. 5A). Therefore, in this tumor model, suppression of *Top1* synergizes with topoisomerase 2 poisoning by chemotherapeutic agents.

**Spontaneous Changes in Topoisomerase Levels Accompany Relapse After Doxorubicin Therapy.** To examine the relevance of topoisomerase status to resistance mechanisms spontaneously occurring in treated lymphomas, primary tumors and postdoxorubicin treatment relapses from Fig. 5A were analyzed for *Top1* and *Top2A* expression levels (Fig. 5B). The relevance of *Top2A* levels to the

emergence of tumor relapses was supported by the fact that half of the relapsed tumors displayed dramatically reduced *Top2A* levels (one of two control tumors and two of four shTop1-expressing tumors) without experimental manipulation via *Top2A* shRNAs. As further evidence that *Top1* knockdown can sensitize to the topoisomerase 2 poison doxorubicin, one shTop1 relapse (relapse 3) recovered expression of *Top1* to approximately wild-type levels. Relapsed tumors treated *ex vivo* showed resistance to doxorubicin, but not cisplatin, suggesting that the resistance mechanisms were topoisomerase-specific (Fig. S9). Together, these results indicate that although alterations in topoisomerase expression levels represent one of undoubtedly many therapy resistance mechanisms, these changes can play a substantial role in chemotherapy response *in vivo*.

## Discussion

In this study we document the utility of combining RNAi screens with mouse cancer models to identify and characterize molecular determinants of therapeutic response that are relevant to treatment outcome *in vivo*. This approach is ideal for rapid *in vivo* validation of candidate genes and may serve as a relevant setting for conducting *in vivo* RNAi-based screens for genetic determinants of drug resistance. Such methodology is easily extendable to other chemotherapeutics and tumor systems to allow a more global view of therapy response mediators, including their context-dependence across different tumor and host genotypes.

The mechanism whereby *Top1* and *Top2A* down-regulation produces resistance to their cognate poisons is probably due to a reduction in topoisomerase–DNA cleavage complexes, resulting in less DNA damage (see Figs. 2D and 4C; ref. 32). By contrast, the mechanism whereby *Top1* down-regulation hypersensitizes to topoisomerase 2 poisons remains to be precisely



viability assays, lymphoma cells were treated in triplicate at the indicated doses of doxorubicin/camptothecin. Viability was determined after 24 h by an FSC versus SSC gate and plotted relative to untreated viability.

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