

The *SIL* Gene Is Essential for Mitotic Entry and Survival of Cancer Cells

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Abstract

Although mitosis is a general physiologic process, cancer cells are unusually sensitive to mitotic inhibitors. Therefore, there is an interest in the identification of novel mitotic inhibitors. Here, we report the novel discovery of the *SIL* gene as a regulator of mitotic entry and cell survival. The *SIL* gene was cloned from leukemia-associated chromosomal translocation. It encodes a cytosolic protein with an unknown function and no homology to known proteins. Previously, we observed an increased expression of *SIL* in multiple cancers that correlated with the expression of mitotic spindle checkpoint genes and with increased metastatic potential. Here, we show that *SIL* is important for the transition from the G₂ to the M phases of the cell cycle. Inducible knockdown of *SIL* in cancer cells *in vitro* delayed entrance into mitosis, decreased activation of the CDK1 (CDC2)-cyclin B complex, and induced apoptosis in a p53-independent manner. *SIL* is also essential for the growth of tumor explants in mice. Thus, *SIL* is required for mitotic entry and cancer cell survival. Because increased expression of *SIL* has been noted in multiple types of cancers and correlates with metastatic spread, it may be a suitable target for novel anticancer therapy. [Cancer Res 2007;67(9):4022–7]

Introduction

SIL was cloned from the most common chromosomal rearrangement in T cell acute lymphoblastic leukemia (1). The *SIL* gene encodes a cytosolic protein with no homology to known proteins that is expressed mainly in proliferating cells (2–4). Upon entrance to the cell cycle, *SIL* is induced in an immediate early fashion, reaches peak levels in mitosis and then degrades upon transition into the next cell cycle (3). Recently, we have shown that *SIL* is phosphorylated during mitosis, a process crucial for its interaction with the mitotic regulator PIN1 (5).

We have observed that *SIL* is expressed in multiple cancers and that its expression correlates with the expression of mitotic spindle

checkpoint genes, with the pathologic mitotic index, and with worse prognosis (6, 7). Here, we show that *SIL* is essential for mitotic entry and the survival of cancer cells *in vitro* and *in vivo*.

Materials and Methods

Plasmids. pTR, pSuper, and pTER were provided by van de Wetering et al. (8). For primers and oligonucleotide sequences, see Supplementary Table S2. For *SIL*-short hairpin RNA (shRNA), the construction of pSuper/pTER was done as published (9). The inducible system was generated by the transfection of pTer-plasmid carrying the tetracycline operator with an insert of shRNA specific for the human *SIL* into LS174T colon cancer cells carrying the tetracycline repressor (8). Two other duplexes of dsRNA oligonucleotides were designed using the BLOCK-iT RNAi designer by Invitrogen (Supplementary Table S1).

Chemical staining. Crystal violet staining was done as published (8). For PAS staining, cells were washed and stained with Schiff's reagent (Merck) according to protocol. For Giemsa staining, cells were incubated with colcemid (Biological Industries) for 16 h, washed with PBS, and subjected to RBC hypotonic lysis buffer (NH₄Cl, 41.5 gm; KNCO₃, 5 gm; EDTA, 1 mL; H₂O, 5 L). Following cytopsin, cells were fixed in May-Grunwald stain, rinsed with water, and stained with Giemsa (Sigma-Aldrich Company) for 7 min.

Cell cycle synchronization and analysis. Prometaphase-synchronized cells were obtained by treating exponentially growing cells with 2.5 mmol/L of thymidine for 17 h, washing twice with PBS buffer, growing them in fresh medium for 9 h, and then re-treating the cells with 2.5 mmol/L of thymidine for 16 h. After 4 h of release, cells were treated with paclitaxel for the indicated times. Cells were analyzed by a two-color flow cytometry using the FACScan (BD Biosciences). For each sample, 5,000 events were collected and analyzed. Antibodies used were antimitotic protein monoclonal (MPM2; Upstate; ref. 10) and FITC-conjugated goat anti-mouse IgG (Biosource). A Roche Diagnostics kit was used for Annexin-propidium iodide (PI) analysis.

Fluorescence *in situ* hybridization probes and procedures. Cells were concentrated by cytopsin and fixed with methanol/acetic acid (3:1). LSI BCR/ABL and LSI BCR/ABL extra-signal dual-color DNA probe kits were used (Vysis). Fluorescence *in situ* hybridization (FISH) was done as developed by Esa et al. (11). Cdc2/cyclin B1 kinase assays were done as previously published (5).

***In vivo* experiments.** All experiments involving mice were approved by the institutional animal care and use committee. Six- to eight-week-old female nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Weizmann Institute) were injected s.c. into the right flanks with 2 × 10⁶ exponentially growing LS174T cells in 200 mL of sterile PBS (pH 7.4). Tumor volume was measured in three dimensions with a caliper. Animals with a tumor burden of >2,000 mm³ were sacrificed. Mice were treated with 2 mg/mL of doxycycline (Sigma) in 2.5% sucrose in their drinking water or with 2.5% sucrose as control, from day 5 after injection of cells. The drinking water was changed twice a week to avoid doxycycline toxic metabolites. After the animals were sacrificed, tumor was removed and stained as described (6).

Results

Inducible shRNA-mediated knockdown of *SIL* arrests the growth of cancer cells *in vitro*. To study the role of *SIL* in cancer

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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cells, we designed an inducible knockdown of SIL in the colon cancer cell line LS174T that stably expresses the tetracycline repressor (8). shRNA is produced after the addition of tetracycline (in the form of doxycycline) to the growth medium. Eight stable clones for the inducible SIL shRNA construct were isolated (Supplementary Fig. S1). To correlate the phenotype with the expression level of SIL, we chose clones with variable levels of SIL after the addition of tetracycline; i.e., clone no. 2 versus no. 6 (Supplementary Fig. S1A). *SIL* expression was down-regulated >6-fold in clone no. 2, whereas there was almost no change in *SIL* levels in clone no. 6.

Exposure of the LS174T clones to tetracycline caused a growth arrest that correlated with the magnitude of *SIL* down-regulation (Fig. 1A). Similar growth suppression was observed in other clones with significant *SIL* knockdown. These results were confirmed by independent transfection of two short interfering RNA (siRNA) oligos (Supplementary Fig. S2) into seven other cancer cell lines: MCF-7 (breast adenocarcinoma), PANC1 (pancreatic carcinoma), U-87 (glioblastoma), Caki-2 (kidney carcinoma), H1299 (non-small cell lung carcinoma), HeLa (cervix adenocarcinoma), and PC3 (prostate adenocarcinoma). Figure 1B is representative of four such cell lines.

We ruled out nonspecific activation of the IFN pathway by shRNA by reverse transcription-PCR of the IFN-inducible gene, OAS1 (ref. 12; Supplementary Fig. S3). To definitely confirm the

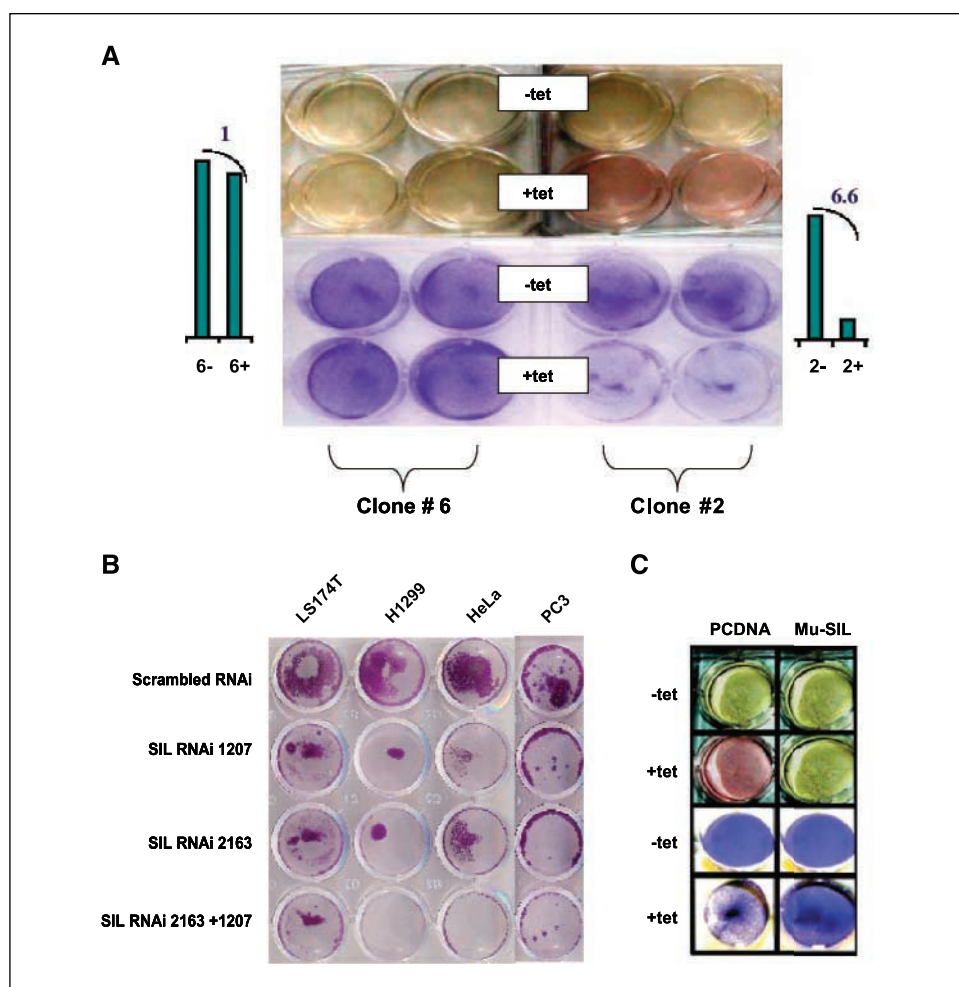
specificity of this phenotype, we transfected clone no. 2 cells with a construct encoding the murine Sil (Mu-Sil), which is not affected by human *SIL* shRNA. After the addition of tetracycline, clones transfected with murine *Sil* survived and proliferated, showing that murine *Sil* rescues the cells from the effects of the knockdown of the endogenous human *SIL* (Fig. 1C). Thus, the inhibition of cell growth is caused by the down-regulation of *SIL*.

SIL knockdown causes apoptosis coupled with a delay in mitotic entry. The phenotype of growth suppression caused by *SIL* knockdown could result from different mechanisms: induction of differentiation, cell death, or perturbation of the cell cycle.

Differentiated colon cells show an increase in the expression levels of the *GAL4* gene (8). There was no change in *GAL4* expression levels after down-regulation of *SIL* (Supplementary Fig. S4). Thus, the difference in growth could not be attributed to increased differentiation. Instead, there was a marked increase in apoptosis after the induction of *SIL* knockdown as depicted in Fig. 2A by a flow cytometry assay using PI and Annexin V. The apoptosis was confirmed independently by a caspase 3 cleavage assay (Fig. 2B).

As the *SIL* expression pattern suggests its involvement in mitosis, we examined the temporal relationship between the apoptosis caused by *SIL* knockdown and the cell cycle. We synchronized the cells at S-G₂ phase by a double thymidine block and treated them with paclitaxel. At different time points, cells

Figure 1. RNAi-mediated knockdown of *SIL* results in a lower number of living cells. **A**, differences in cell growth before (–Tet) and after (+Tet) the addition of tetracycline. **Right**, clone no. 2, in which *SIL* is down-regulated six times more than in clone no. 6 (columns). Similar results were seen with additional clones (data not shown). The difference in growth is shown by the change of the color of natural medium in the plate containing more cells and by the complementary crystal violet staining. **B**, the effect of *SIL* knockdown on the growth of several cancer cell lines transfected with two different siRNA oligonucleotides for *SIL* (oligos 1207 and 2163, see Supplementary Table S2). As a control, a scrambled RNAi was used. H1299 (non-small cell lung cancer, carcinoma), HeLa (cervical adenocarcinoma), PC3 (prostate adenocarcinoma), and LS174T (colon carcinoma) were used. Similar results were observed with additional cell lines: MCF-7 (breast adenocarcinoma), PANC1 (pancreatic carcinoma), U-87 (glioblastoma), and Caki-2 (kidney carcinoma). **C**, murine *Sil* rescues the human *SIL* knockdown: natural medium and crystal violet staining of clone no. 2 posttransfection with either pcDNA3 or murine *SIL* with or without tetracycline exposure.



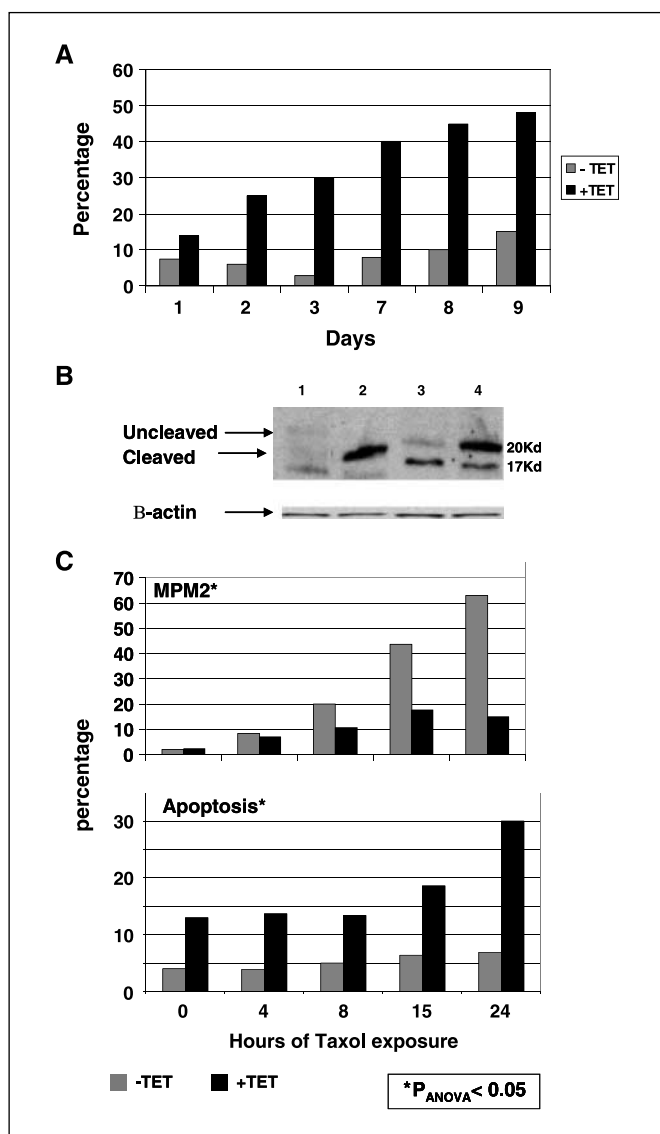


Figure 2. SIL down-regulation causes apoptosis. **A**, flow cytometry analysis for Annexin-PI comparing the percentage of apoptosis (Annexin+, PI-) before (-) and after (+) the addition of tetracycline. This graph is a representative assay of three independent experiments. **B**, a Western gel of caspase 3 cleavage products; when apoptosis occurs, the 20 kDa band diminishes and the 17 kDa band appears as evidence of caspase 3 cleavage. 1, positive control (apoptotic Baf3 cells-murine pro-B cells, IL-3-dependent cells, grown without IL-3); 2, negative control-living cells; 3, synchronized cells exposed to tetracycline and paclitaxel for 24 h; 4, synchronized cells exposed to paclitaxel without tetracycline. The loading control was β -actin. **C**, the delay in mitotic entry in SIL knockdown cells is associated with increased apoptosis. *Left*, percentage of MPM-2 positive (mitotic) cells before and after the addition of tetracycline (Tet). *Right*, percentage of apoptotic cells as calculated from the sub-G₁ fraction. Paclitaxel was added 4 h after release from double thymidine block. This is a representative experiment from 10 similar ones.

were harvested and analyzed both for mitosis by staining with the MPM2 antibody (a mouse monoclonal antibody specific for phosphorylated mitotic proteins) and for apoptosis by measurement of the sub-G₁ fraction by staining with PI (Fig. 2C). SIL knockdown almost completely prevented mitotic entry coupled with a marked increment of apoptosis upon increasing exposure times to paclitaxel. Thus, it seems that apoptosis was associated with an impediment in G₂ to M transition.

We next studied, in more detail, the effects of SIL knockdown on the mitotic phenotype. Metaphase cell spreads were prepared from LS174T clones exposed to colchicine in the presence of tetracycline (Fig. 3A). There was almost no metaphase in cells from clone no. 2 in which SIL expression was eliminated (panel 3) compared with normal cells or clone no. 6 in which SIL is expressed (panels 1 and 2).

To observe mitoses biochemically, we used the antibody MPM2, which allows the distinction between the G₂ fraction and the M fraction of the cell cycle which are usually combined in the standard flow cytometry analyses of DNA content. As seen in a representative experiment (Fig. 3B), a smaller but still considerable percentage of the SIL knockdown cells had DNA content consistent with either the G₂ or M phases (*top, histograms*); however, a true mitotic fraction was nearly absent in SIL knockdown cells, as measured by anti-MPM2 antibody (*bottom, dot plots*). This strongly suggests that in the absence of SIL, the cells do not enter the M phase and undergo apoptosis in the G₂ to M boundary (Fig. 2C).

To further analyze this mitotic phenotype, we did a FISH assay on clone no. 2 with probes targeting the *BCR* and *ABL* genes on chromosome 22 and 9, respectively. Cells were exposed to colchicine for 16 h before and after treatment with tetracycline. In correlation with the results above, the majority of SIL knockdown cells were not in mitosis but displayed a clear pattern of doublet signal hybridization, characteristic of the G₂ phase (Fig. 3C).

The major regulator of mitotic entry is the CDK1 (CDC2)-cyclin B complex. To test if the perturbed mitotic entry of SIL knockdown cells is associated with a decreased activity of CDK1, we did a kinase assay for its activity. To arrest cells at metaphase, cells were treated with paclitaxel alone or with paclitaxel and tetracycline. Cell lysates were collected at different time points and the CDK1-cyclin B complex was immunoprecipitated with anti-cyclin B1 antibody. Consistent with the delay in mitotic entry, there was a marked delay in the activation of CDK1 in SIL knockdown cells (Fig. 3D). Thus, cells with low levels of SIL display a marked decrease in mitotic entry associated with the low activity of CDK1.

To further examine the specificity of the shRNA-mediated knockdown of the human SIL, we analyzed the cell cycle and apoptosis patterns of clone no. 2 cells stably expressing the Mu-Sil. The ectopic expression of Mu-Sil rescued these cells from apoptosis and G₂ arrest. Thus, both phenotypes of delayed mitotic entry and the apoptotic phenotype are specifically caused by SIL down-regulation (Supplementary Fig. S5A and B).

SIL is required for cancer growth *in vivo*. To investigate the requirement of SIL for tumor growth *in vivo*, we s.c. injected immunodeficient NOD/SCID mice with 2×10^6 cells of LS clone no. 2. Tetracycline was added to the drinking water 5 days after the injections to half of the mice. The growth of the tumors in the tetracycline group was significantly slower than in the untreated group (Fig. 4A and B). As expected, there was no effect of treatment with tetracycline on the growth of tumors derived from clone no. 6, in which SIL shRNA has nearly no effect on SIL levels (Supplementary Fig. S6).

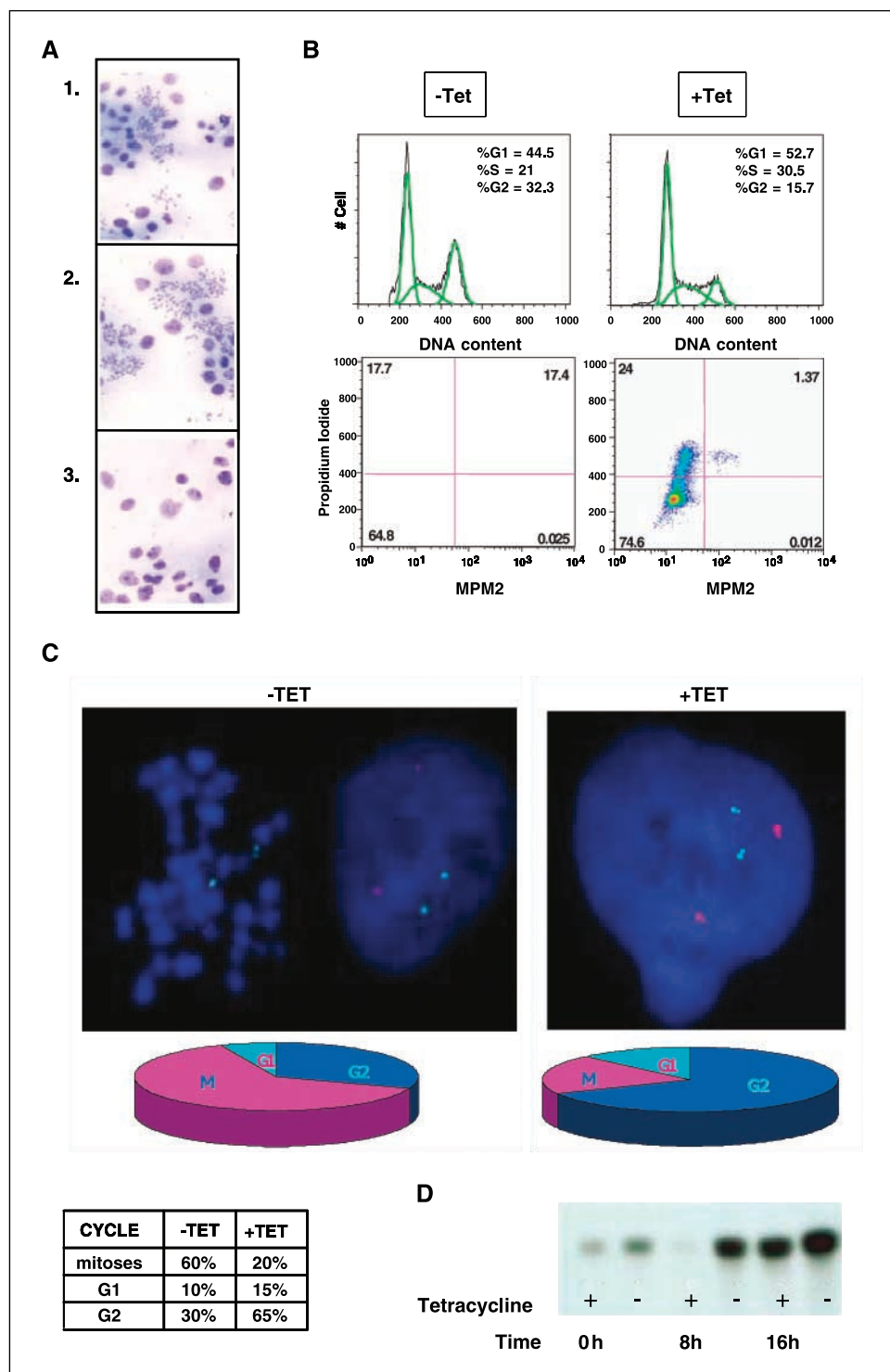
After 3 weeks of continuous exposure to tetracycline, several clone no. 2 tumors began to grow at the same doubling time as those without tetracycline exposure. This escape could have been explained either by selection of cells in which there is no shRNA effect on SIL or by an acquired mutation promoting cell

growth despite the continuous reduction of SIL. To distinguish between these possibilities, we analyzed the tumors for SIL mRNA and protein levels. As shown in Fig. 4C and D, SIL was expressed in the growing tumors, suggesting that SIL shRNA was not expressed or was not active in these cells. Thus, the tumors grew only after escaping the RNAi-mediated SIL repression, suggesting that SIL is critical for cell growth and survival.

Discussion

In this study, we have shown the requirement of SIL for cancer cell survival and mitotic entry. To elucidate the role of SIL in cancer cells, we generated an inducible knockdown of the endogenous human SIL. We show that SIL is necessary for the survival of L174T colon cancer cells *in vitro* and *in vivo*, in a manner that correlates with its expression levels. Interestingly, examination of explant tumors in mice that escape the growth

Figure 3. Inhibition of mitotic entry in the absence of SIL. **A**, metaphase spreads after exposure to colchicine for 16 h in the presence of tetracycline; (Giemsa staining) untransfected LS174T (1), clone no. 6 (2), and clone no. 2 (3; magnification, $\times 40$). **B**, flow cytometry analysis of cells stained for PI and MPM2 with or without tetracycline; *top*, DNA content; *bottom*, dot plots obtained by costaining with anti-MPM2 and PI. The sub-G₁ phase was eliminated in these graphs. **C**, FISH analysis after 16 h of exposure to colchicine using probes for ABL (chromosome 9; *red*) and BCR (chromosome 22; *green*; magnification, $\times 100$); *Top left*, clone no. 2 cells in the absence of tetracycline; *right*, after the addition of tetracycline. *Bottom*, cell cycle distribution determined by counting 200 cells. **D**, kinase assay for CDK1 activity following exposure of the cells, with or without tetracycline (*Tet*), to paclitaxel. Whether or not tetracycline was added is indicated along with the time following exposure to paclitaxel.



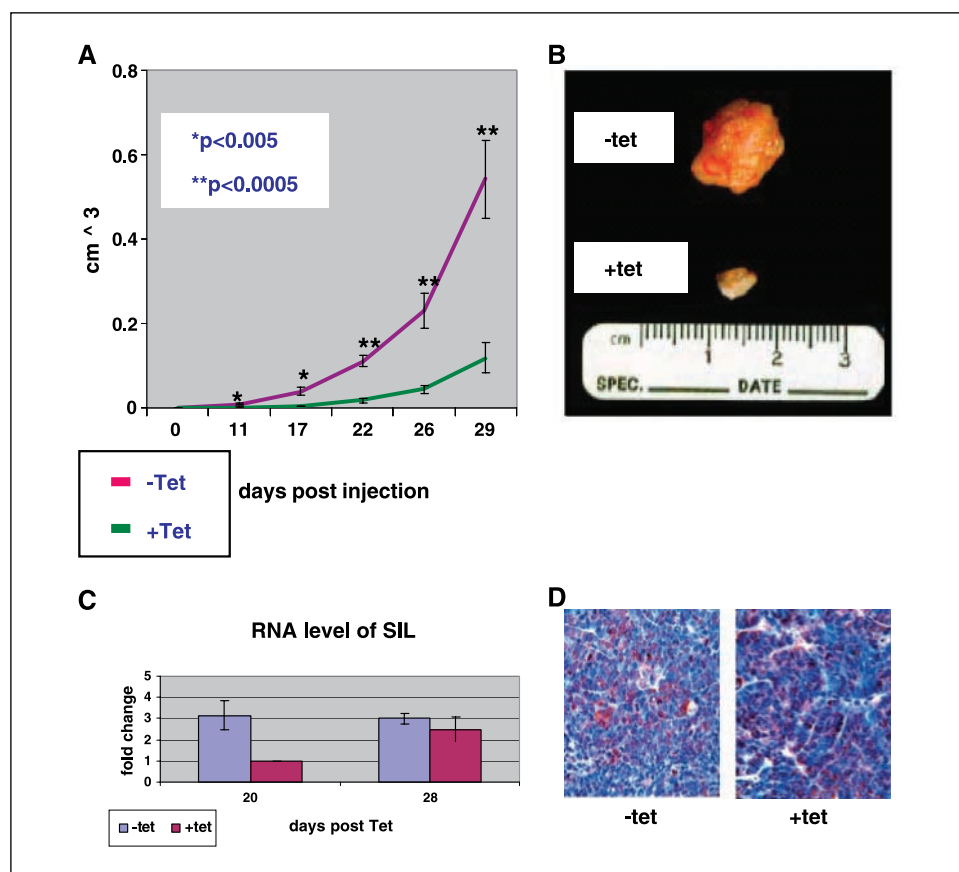


Figure 4. SIL down-regulation prevents tumor growth *in vivo*. **A**, 20 female mice were injected with 2×10^6 LS2 cells. Five days postinjection, half of the mice received tetracycline in their drinking water. Every 3 d, the water was changed to avoid tetracycline degradation. Twice a week, tumor size was measured in all mice in three diameters ($X \times Y \times Z / 2$). The graph shows the average growth in each group in three different experiments. A *t* test was used to analyze each group on each measurement day. **B**, two examples of tumors excised from mice in which one received Tet and the other did not. **C**, real-time PCR results for SIL RNA levels in tumors excised at days 20 and 28. **D**, immunostaining for SIL in tumors excised from mice at day 28 showing SIL expression in tumors that escaped the growth arrest.

suppression of tetracycline revealed normal expression of SIL. These findings suggest that SIL is essential for tumor growth and that the tumor escape is caused by “takeover” of cells in which the shRNA was probably silenced or lost. The apoptosis induced in the absence of SIL does not depend on an intact p53 pathway as it is also observed in p53-deficient cell lines transfected with the SIL-specific siRNA oligos.

In addition to the induction of apoptosis, SIL knockdown resulted in a substantial reduction of mitotic entry. Using morphologic, FISH, and biochemical analyses we showed that in the absence of SIL, cells delay their entry to mitosis and undergo apoptosis. This phenotype was most pronounced upon treatment with chemical agents that cause metaphase arrest (colchicine and paclitaxel). SIL is also necessary for the timely activation of CDK1 (CDC2), the major kinase regulating mitotic entry (13, 14). SIL may either directly regulate the activation of CDK1 or it may work further upstream. The rescue of the knockdown phenotype by a construct encoding murine Sil confirms the specificity of the knockdown and provides a useful system to further characterize the structural elements in SIL mediating these mitotic and apoptotic phenotypes.

We have recently shown that SIL is phosphorylated during mitosis on several conserved serine/threonine residues (5) necessary for its interactions with the mitotic regulator PIN1. Because the phosphorylation occurs during mitosis, it is probably not required for regulation of mitotic entry. This conclusion is strengthened by preliminary experiments which show that SIL mutated in the phosphorylation sites rescues the apoptosis phenotype of LS174T cells with knockdown of the endogenous

SIL (Castiel and Izraeli; data not shown). Therefore, we believe that mitotic phosphorylation of SIL is not important for its pro-survival function at mitotic entry.

We have reported here our novel discovery that SIL functions in a “checkpoint” coupling the transition into mitosis with cell survival in variety of cancer cells. SIL is not necessary for the survival of all cells. Mouse embryonic stem cells lacking any functional Sil protein proliferate normally and create teratomas in nude mice (4). Although mitosis is a general physiologic process, cancer cells are highly sensitive to antimitotic drugs. Indeed the therapeutic ratio of drugs such as paclitaxel and vincristine is surprisingly high. Consequently, there is a marked effort to develop new drugs targeting molecules that regulate mitosis and mitotic entry (15, 16). As the SIL protein regulates mitotic entry and cell survival, it may prove to be a target for novel anticancer therapeutics.

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References

1. Aplan PD, Kirsch IR. Structural characterization of SIL, a gene frequently disrupted in T-cell acute lymphoblastic leukemia. *Mol Cell Biol* 1991;11:5462-9.
2. Golling G, Amsterdam A, Sun Z, et al. Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development. *Nat Genet* 2002;31:135-40.
3. Izraeli S, Colaizzo-Anas T, Bertness VL, Mani K, Aplan PD, Kirsch IR. Expression of the SIL gene is correlated with growth induction and cellular proliferation. *Cell Growth Differ* 1997;8:1171-9.
4. Izraeli S, Lowe LA, Bertness VL, et al. The SIL gene is required for mouse embryonic axial development and left-right specification. *Nature* 1999;399:691-4.
5. Campaner S, Kaldis P, Izraeli S, Kirsch IR. Sil phosphorylation in a Pin1 binding domain affects the duration of the spindle checkpoint. *Mol Cell Biol* 2005;25:6660-72.
6. Erez A, Perelman M, Hewitt SM, et al. Sil overexpression in lung cancer characterizes tumors with increased mitotic activity. *Oncogene* 2004;23:5371-7.
7. Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of metastasis in primary solid tumors. *Nat Genet* 2003;33:49-54.
8. van de Wetering M, Oving I, Muncan V, et al. Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. *EMBO Rep* 2003;4:609-15.
9. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002;296:550-3.
10. Andreassen PR, Margolis RL. Microtubule dependency of p34cdc2 inactivation and mitotic exit in mammalian cells. *J Cell Biol* 1994;127:789-802.
11. Esa A, Trakhtenbrot L, Hausmann M, et al. Fast-FISH detection and semi-automated image analysis of numerical chromosome aberrations in hematological malignancies. *Anal Cell Pathol* 1998;16:211-22.
12. Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R. Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet* 2003;34:263-4.
13. Ferrari S. Protein kinases controlling the onset of mitosis. *Cell Mol Life Sci* 2006;63:781-95.
14. Riabowol K, Draeta G, Brizuela L, Vandre D, Beach D. The cdc2 kinase is a nuclear protein that is essential for mitosis in mammalian cells. *Cell* 1989;57:393-401.
15. Weaver BA. Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death. *Cancer Cell* 2005;8:7-12.
16. Rajagopalan H, Lengauer C. Aneuploidy and cancer. *Nature* 2004;432:338-41.