IN1 Induces Interferon Signaling and Spindle Checkpoint in Rhabdoid Tumors

Alexei Morozov,1 Seung Jae Lee,1 Zhi-Kai Zhang,1 Velasco Cimica,1 David Zagzag,2 and Ganjam V. Kalpana1

Abstract Purpose: Rhabdoid tumors are rare but aggressive pediatric malignancies characterized by biallelic loss of IN1/hSNF5. Reintroduction of IN1 causes cell arrest and senescence in rhabdoid cells. Our purpose was to identify IN1-downstream genes and to determine their functional and therapeutic significance for rhabdoid tumors.

Experimental Design: IN1 downstream targets in rhabdoid cells were identified using a cDNA microarray analysis and the expression of selected IN1 targets was confirmed by quantitative reverse transcription-PCR, Western analysis, and/or immunohistochemical analysis of rhabdoid cells and primary rhabdoid tumors. To determine the functional significance of downstream targets, activated targets of IN1 were induced and repressed targets of IN1 were knocked down (by using RNA interference) in rhabdoid cells, in the absence of IN1. Consequence of altered expression of IN1 downstream targets for rhabdoid cell survival, cell cycle, and apoptosis was assessed.

Results: Microarray studies indicated that IN1 activated IFN-stimulated genes at early time points and senescence markers at late time points and repressed mitotic genes such as Polo like kinase 1 (PLK1), selectively in rhabdoid cells. Treatment of rhabdoid cells with recombinant IFNs resulted in induction of IFN-stimulated genes, G1 arrest, and flat cell formation. PLK1 was overexpressed in primary human and mouse rhabdoid tumors. RNA interference—mediated knock down of PLK1 in rhabdoid cells resulted in mitotic arrest, aberrant nuclear division, decreased survival, and induction of apoptosis.

Conclusions: Targeting downstream effectors of IN1 such as IFN pathway and mitotic genes leads to antiproliferative effects in rhabdoid cells. IFN treatment and down-modulation of PLK1 constitute potential novel therapeutic strategies for rhabdoid tumors.

Rhabdoid tumors of the kidney (malignant rhabdoid tumors), brain (atypical teratoid and rhabdoid tumors), and soft tissues are rare, aggressive, and incurable pediatric malignancies (1, 2). Currently, there are no effective or standard treatment strategies for these tumors. Prognosis for these tumors is poor despite the use of potent chemotherapeutic, radiotherapeutic, and surgical interventions (1–3). Thus, there is a dire need to develop novel therapeutic strategies, based on the sound understanding of the specific molecular alterations responsible for pathogenesis of these tumors. IN1 (also known as hSNF5, BAF47, SMARCBl) is a tumor suppressor biallelically deleted/mutated in >85% of rhabdoid tumors (4). Mouse knockout and human cytogenetic studies have established that despite the divergent tissue of origin, biallelic alteration of IN1 gene is a critical pathogenic event that leads to rhabdoid tumor formation (5–8). These studies also showed that rhabdoid tumors are developed due to loss of heterozygosity in IN1 gene. IN1 was first isolated as an interacting protein for HIV-1 integrase and is a component of the evolutionarily conserved human SWI/SNF complex that remodels the chromatin in an ATP-dependent manner (9, 10). Components of SWI/SNF complex are involved in either activation or repression of only a subset of genes (11).

Because IN1 inactivation/deletion is a critical event in the transformation of rhabdoid tumors, we surmised that identifying functionally significant downstream effectors of IN1/hSNF5 and pathways altered in rhabdoid tumors would lead to the development of novel therapeutic strategies for these tumors. Previously, we identified cyclin D1 as critical gene directly repressed by IN1 (12–14). Genetic knockout studies indicated that cyclin D1 is required for genesis of rhabdoid tumors in vivo. Knockdown of cyclin D1 by RNA interference in rhabdoid cells indicated its requirement for rhabdoid cell survival (12, 13). In addition, therapeutically targeting cyclin D1 inhibited rhabdoid cell growth in vitro and in vivo (12).
These studies showed that identifying functionally significant INI1 downstream effectors is a useful strategy in developing potential targeted therapies for rhabdoid tumors. The aggressive nature of rhabdoid tumors and its insensitivity to many potent chemotherapy regimens suggest the deregulation of multiple cell cycle and growth control pathways in these tumors. In this report, we sought to identify additional genes and pathways that are downstream of INI1 in rhabdoid cells, and assess their usefulness as therapeutic targets for rhabdoid tumors. For this purpose, we carried out cDNA microarray analysis to determine the expression changes of genes in rhabdoid cells upon reintroduction of INI1. Previous studies have analyzed INI1-induced gene expression changes at early time points in rhabdoid cells (15, 16). Others have explored the INI1-dependent expression profile changes in the nonrhabdoid mouse embryo fibroblasts (17). Neither of these studies explored the possibility of using downstream effectors as candidate therapeutic targets. Here, we report the identification of several downstream pathways of INI1 that are candidate therapeutic targets. We found that (a) a high frequency of INI1-responsive genes are induced at early time points; (b) tissue-specific and senescence-associated genes are up-regulated at latter time points; and (c) mitotic spindle check point genes, such as Polo like kinase 1 (PLK1), are down-regulated. We further show that INI1 treatment and down-modulation of PLK1 leads to inhibition of rhabdoid cell growth and induction of cell cycle arrest. Our studies suggest that these pathways could be explored further for developing novel targeted therapies for rhabdoid tumors.

Materials and Methods
cDNA microarray analysis. For the 11-day time point, MON cells (5 × 10⁶) were transfected with either pEGFP-N1 (Clontech) or pEGFP-N1-INI1 encoding a green fluorescent protein (GFP)-INI1 fusion protein and selected for neomycin (18). For the 3-day time point, MON cells were cotransfected with a mixture of 10-fold molar excess of plasmids either expressing GFP-INI1 or GFP along with the plasmid expressing puromycin resistance gene. The transfected cells were selected for 3 days in puromycin. To analyze INI1-induced gene expression changes after 24 h of transfection, MON cells were transfected with GFP or GFP-INI1 by calcium phosphate precipitation. The GFP-positive cells were sorted after 6 h after medium change, which was 24 h posttransfection, using FACSort. Cells (2 × 10⁵ to 5 × 10⁵) were routinely obtained after 2 h of sorting. One hundred micrograms RNA each from GFP-INI1 and GFP-transfected cells were used from the 1- and 3-day time points. About 10 μg of RNA isolated from the 24-h time point was amplified using MessageAmp aRNA kit (Ambion). GFP-INI1 and GFP samples were converted into cDNA while incorporating Cy3-dUTP or Cy5-dUTP.

The labeled cDNAs were mixed and hybridized to 9,000, 18,000, 26,000 or 29,000 spot human cDNA microarray prepared in-house (depending on the availability at the time of experimentation). Although different number of spots, all arrays include subset of genes from all functional groups and hence are representative. The entire experiment was repeated starting with a new frozen vial of cells. Different batches of plasmid DNA, different series of microarrays, and different combinations of dyes were used each time. The chips were scanned using a GenePix laser scanner and the data further processed using the BioArray Software Environment. Normalization was done according to the Lowess algorithm (19).

Cell lines used. MON and STA-WT1 are rhabdoid cell lines defective for INI1 (4). Nonrhabdoid cell lines include HeLa (Sigma), 293T (American Type Culture Collection), Glioma SF268 (kind gift of Dr. Agus, Albert Einstein College of Medicine, Bronx, NY), and Jurkats (NIH AIDS research reagents and repository).

IFN receptor blocking. The cells were transfected with either GFP-INI1 or GFP and 1 μg/mL monoclonal α-hIFN-γR1 antibody (R&D Systems) or 5 μg/mL monoclonal α-hIFNα/βR2 antibody (Biomedical Labs) were added to the cells at the time of medium change preceding transfection for 72 h. RNA was prepared from the cells and analyzed by real-time reverse transcription-PCR (RT-PCR).

Induction of flat cell morphology by recombinant IFN. MON cells were seeded at 10% confluency in six-well plates with 0, 1,000, 2,000, or 4,000 IU/mL recombinant human IFN-γ (R&D Systems) or 0, 100, 200, or 400 IU/mL human IFN-γ (Biomedical Laboratories). Confluency was estimated daily in a blinded fashion. The cells were passaged when they reached near confluency. After 2 weeks, the cells were photographed in a blinded fashion.

Preparation of retroviral vectors and infection. Retroviral vectors expressing INI1-Flag were generated in 293T producer cells by transfecting three plasmids: (a) pBabe-FLAG or pBabe FLAG INI1; (b) pMLV/GagPol, expressing Gag Pol; and (c) pMDG, expressing VSVG envelope, using calcium phosphate precipitation method (20). The retroviral vector was used to infect 30% to 40% confluent MON or STA-WT1 cells for 9 h, and the transduced cells were selected with puromycin (1 μg/mL) for various time points.

RT-PCR analysis of PLK1. Total RNA was isolated from transfected MON cells 4 to 6 days of infection using RNeasy kit (Qiagen). RT-PCR analysis was done using Access RT-PCR system (Promega) as per manufacturer’s recommendation, using primer to amplify PLK1 (forward: 5′-CGGACGGGACAGATTGCTCA-3′, reverse: 5′-AATACCTGGTTTCGGTGACAG-3′; ref. 21) or glyceraldehyde-3-phosphate dehydrogenase (forward: 5′-TGGAACCCCATCACTCCT-3′, reverse: 5′-TTGACACCA-TGCGAACATC-3′) cDNAs using 23 cycles.

Immunoblot analysis. Western blot analysis was carried out with following antibodies: anti-FLAG M2-peroxidase (horseradish peroxidase) conjugate (Sigma), anti-PLK1 (Upstate), α-tubulin antibody (Sigma), and α-IN1 antibody prepared in-house (PB3; ref. 13).

Small interfering RNA analysis. The cells seeded in a six-well plate at a density of 3 × 10⁵ per well were transfected with 100 nmol/L of each small interfering RNA (siRNA) using Dharmafect 1 (Dharmacon) according to the manufacturer’s instructions. The following siRNAs were purchased from Dharmacon Research, Inc.: PLK1 (AACCAGUG-GUUCGAGAGACAGUU; ref. 22), with two thymidineresides (dTdT) at the 3′ end (23), siINI1 (Dharmacon Smartpool siRNA duplex SMARB1), and cy3-luciferase GL2 Duplex (Dharmacon) as a control.

Immunohistochemical analysis. MON cells transfected with siRNA were subjected to immunohistochemical analyses as reported before (22). Antigen retrieval was carried out in citrate buffer and 1:100 dilution of α-PLK1 primary antibody (Zymed) was used for 2 h at room temperature. Staining was achieved using avidin-biotin system following the manufacturer’s instruction (Vector Laboratories M.O.M.). The results of immunohistochemical analyses were verified by the pathologist author (D.Z.).

Cell cycle, cell proliferation, and caspase-3/7 assays. Cell cycle analysis was carried out as previously described (14) with minor modification. MTS (3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) and caspase assays were done using MTS assay kit (Cell Titer 96 AQ Non-Radioactive Cell Proliferation kit, Promega) and caspase-Glo-3/7 assay kit (Promega).

Results
INI1/hSNF5 up-regulates differentiation and senescence genes and down-regulates mitotic genes. To determine the downstream targets of INI1 in rhabdoid tumor cells, we carried out a cDNA microarray analysis, comparing the temporal expression profile in cells transfected with GFP to those transfected with GFP-INI1. We chose three different time points that reflected
The microarray results were further confirmed by quantitative real-time PCR analysis, using RNA isolated from a fresh batch of transfected cells (Table 1).

### Table 1. Independent validation of induction of senescence/differentiation markers and IFN-stimulated genes by INI1

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<th>Symbol</th>
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<th>Average fold change on chip*</th>
<th>Average fold change by quantitative RT-PCR</th>
<th>IFN signal</th>
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Abbreviations: UC, unchanged (no change in fold expression on cDNA microarray); NC, not present on the chip; ND, not determined.

* Fold change of INI1-induced gene expression, as determined using microarrays.

* Fold change of INI1-induced genes as determined by quantitative real-time PCR, using RNA from a freshly transfected batch of cells.

**Induction of IFN signal by INI1 in MON cells.** Unlike at the 11-day time point, we found that there was an overwhelming, statistically significant ($P < 0.001$) abundance of well-known IFN-stimulated genes at early time points of 24 h and 3 days (Supplementary Table S2; ref. 30). We found that at the 72-h time point, 109 and 16 genes were up- and down-regulated $>1.8$-fold in two of three experiments. At the 24-h time point, 37 and 4 genes were up- and down-regulated, respectively, in two of two experiments. Overall, 20 of 34 activated genes at the 24-h time point, and 15 of 109 activated genes for the 3-day time point were known IFN-stimulated genes. There was also a significant overlap between IFN-stimulated genes activated at 24 h and 3 days (Supplementary Table S2). There was no overlap and no IFN-stimulated genes among the genes down-regulated at the 3- and 1-day time point (data not shown). These results established that in rhabdoid cells, INI1 induces transcription of IFN-stimulated genes at early time points. These results are consistent with the previous findings that BRG1 and INI1 are necessary for inducing IFN-stimulated genes in HeLa cells (31, 32).

Several observations validated our microarray analysis. First, there was significant overlap between genes activated at 3 and 11 days (Supplementary Table S2), as well as 24 h and 3 days (Supplementary Table S2). Second, several activated genes were represented by duplicate spots with similar fold changes (e.g., IFITM1, SP110, G1P2, IFITM3; Supplementary Table S2). In addition, we confirmed the activation of selected genes by quantitative RT-PCR (Table 1).

**Signaling through the IFN receptor is necessary for the induction of IFN target genes by INI1.** To determine if induction of IFN-stimulated genes by INI1 is mediated by IFN signaling, we did an IFN receptor blocking experiment. Real-time RT-PCR was used to assess the effect of IFN blocking antibody in INI1-mediated induction of an IFN target, LGALS3BP (galectin 3 binding protein), and another INI1 up-regulated gene not known to be an IFN target, MMP1.
(Fig. 1A). As expected, both genes were activated in GFP-INI1-transfected cells compared with GFP-transfected cells (Fig. 1A). However, in the presence of either type I or type II receptor blocking antibodies, there was a significant reduction in the activation of these genes. These results indicated that up-regulation of known IFN-stimulated genes and other genes by INI1 in MON cell require IFN signaling.

Type I or type II recombinant IFNs are able to partially bypass INI1 requirement in inducing many INI1 target genes. Recent studies implicate SWI/SNF complex in IFN signaling in two ways. First, it is required for induced level expression of IFN-stimulated genes (31). This finding is consistent with our results that INI1 activates IFN target genes. Second, SWI/SNF complex was required for the induced expression of IFN genes themselves (33). Based on these reports, we hypothesized that INI1 may be required for induction of IFN signaling at two levels—one at the level of induction of type I or type II IFN genes and second at the level of induction of IFN-stimulated genes. We further hypothesized that the requirement of INI1 could be partially bypassed by the addition of IFN due to increased signaling by IFNs to IFN-stimulated gene promoters. If this were to be true, this could be potentially interesting from...
the therapeutic point of view. We may be able to induce the IFN target genes by the addition of exogenous IFNs, by increasing signaling, to partially mimic the effects of INI1 in rhabdoid cells.

To test the hypothesis that addition of exogenous IFN may increase the signaling in the absence of INI1, we examined expression levels of a panel of INI1 up-regulated genes in MON cells following IFN-γ or IFN-β treatment for 3 days in the absence of INI1 using quantitative RT-PCR (Fig. 1B and C). We found that recombinant IFNs are sufficient to induce expression of a subset of INI1 up-regulated genes that are IFN targets (Fig. 1B and C, left shaded area). However, there was variability in induction of genes not known to be IFN targets in that some genes were up-regulated by addition of IFNs and some were not (Fig. 1B and C, right). Nevertheless, these results indicated that reintroduction of INI1 or addition of extracellular IFN at least partially relieves a block to IFN signaling in rhabdoid cells.

Type I or type II recombinant IFN is sufficient to induce flat cell formation in the absence of INI1. IFN treatment is an approved therapy and has been used as a treatment strategy for a number of neoplasms (Supplementary Table S3). IFNs have been shown to induce antiproliferative effects in various cancer cell lines (34–36). Furthermore, induction of IFN-stimulated genes has been shown to be associated with inhibition of immortalization and induction of senescence-like phenotype in certain cancer cells (37–39). Because addition of exogenous IFN itself induced many of the INI1-induced target genes, we tested to determine if this treatment could induce either cell cycle arrest or flat cell formation in the absence of INI1 in rhabdoid cells.

We found that IFN treatment resulted in decrease in S phase (P < 0.00268) and G1 arrest in MON cells as determined by fluorescence-activated cell sorting analysis (Fig. 1D). To determine the long-term effect of IFN treatment, the MON and STA-WT1 cells were plated at ~10% confluency in the absence or presence of IFN-γ or IFN-β at concentrations known to induce differentiation of other tumor cell lines (40, 41). We found that 5 to 7 days after one round of replating, the cultures treated with IFN-γ or IFN-β showed reduced cell numbers compared with no-IFN controls (Fig. 1D). In addition, whereas few or no flat cells were observed in control cultures, significant increase in flat cells were found among IFN-treated cells (Fig. 1D and E). We conclude from these results that IFN is sufficient to induce cell cycle arrest and flat cell formation in the absence of INI1 in rhabdoid cells.

Down-modulation of PLK1 by INI1/hSNF5 specifically in rhabdoid cells. The effect of INI1 is mediated both by up- and down-regulating expression of key cellular genes. We found that an overwhelming majority of genes down-regulated by INI1 are involved in mitosis (Supplementary Table S1B). Among these down-regulated genes were those that could be potential therapeutic targets, such as PLK1.

PLK1, a crucial mitosis-promoting kinase, is currently an attractive target for developing anticancer therapy (42). Overexpression of this protein has been observed in many tumors and is a prognostic marker in some cases (43). Overexpression of PLK1 is associated with aggressive stage and poor survival in cancer patients (43). Expression of PLK1 seems to be tightly regulated by many tumor suppressors, including Chk1 and BRCA1, to prevent transformation (43). Furthermore, it seems that whereas tumor cells are sensitive to down-modulation of PLK1 due to induction of mitotic catastrophe, normal diploid cells seems to tolerate its inhibition, possibly due to the presence of redundant mechanisms in normal cells (22, 44). These studies indicate that PLK1 is a potential target for developing anticancer strategies. Therefore, we hypothesized that inhibiting PLK1 in rhabdoid cells could be effective in inhibiting the growth of these cells.

As a first step, we carried out semiquantitative RT-PCR and immunoblot analyses to confirm the microarray results by using a fresh batch of MON cells that were transduced either with an empty retroviral vector or vector expressing INI1. The results showed that INI1 down-modulated PLK1 at both mRNA and protein levels, confirming the microarray results (Fig. 2A and B).

Loss of INI1 is specific to rhabdoid tumors. However, INI1 is present in most other tumor types. Because PLK1 up-regulation is not specific to rhabdoid cells, we determined the specificity of PLK1 repression by INI1 in various tumor cell lines. We generated transient pools of isogenic pairs of cells differing from each other in the expression of INI1. For this purpose, either INI1 was reintroduced into INI1-lacking rhabdoid cells or it was knocked down from INI1-containing nonrhabdoid cells by RNA interference. Protein isolated from cells with or without INI1 was subjected to immunoblot analysis to determine the expression of PLK1. We found that reintroduction of INI1 into STA-WT1 cell line, another rhabdoid tumor cell line, also resulted in PLK1 down-modulation (Fig. 2C). In nonrhabdoid cells (HeLa, SF268 glioma, Jurkats, and 293T cells), successful siRNA-mediated down-modulation (>80%) of INI1 was observed. Intriguingly, in nonrhabdoid tumor cell lines, down-modulation of INI1 had no effect on PLK1 expression (Fig. 2D). Thus, it seems that although PLK1 is expressed in many tumor cell lines, its repression by INI1 is specific to rhabdoid cells. In other words, whereas presence of INI1 in nonrhabdoid tumor cell lines does not affect expression of PLK1, its presence in rhabdoid cells represses PLK1.

Rhabdoid cells overexpress PLK1. We next surmised that if INI1 represses PLK1 expression, rhabdoid tumors deleted of INI1 should exhibit up-regulation of this protein. We carried out immunohistochemical analysis of four human and one mouse primary rhabdoid tumors (developed in an Ini1 knockout mouse in our laboratory) to determine if PLK1 is up-regulated. These primary tumors were previously shown to have all characteristics of rhabdoid tumors (13, 14). The result of this analysis showed the presence of strong positive cytoplasmic signal with PLK1 antibody in all the rhabdoid tumors (Fig. 2E and F). The staining was also evident within the rhabdoid cells, which are characterized by the presence of eccentric nuclei and large nucleoli (Fig. 2F, bottom). Contrary to the strong staining of all the tumor cells, normal brain cells and stromal cells within the rhabdoid tumors were negative for PLK1 expression (Fig. 2D and F). These results, for the first time, indicated that rhabdoid tumors overexpress PLK1.

Down-modulation of PLK1 leads to aberrant mitotic segregation in rhabdoid cells. Inhibiting PLK1 is currently an attractive cancer therapy that has shown promise in preclinical studies (43, 45). Down-modulation of PLK1 by INI1 and overexpression of this protein in human and mouse rhabdoid tumors suggested that it is a potential therapeutic target for rhabdoid tumors as well. To test this concept, we used RNA interference to down-modulate PLK1 in rhabdoid cells. Expression of PLK1-specific siRNA resulted in down-modulation of PLK1 protein.
Repression of PLK1 by INI1 specifically in rhabdoid cells and its overexpression in human and mouse rhabdoid tumors. A. Semiquantitative RT-PCR analysis to determine repression of PLK1 by INI1 in MON cells. Total RNA was isolated from cells infected with control or INI1-Flag expressing retroviral vectors and subjected to RT-PCR analysis. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B and C, Immunoblot analysis to show down-modulation of PLK1 by INI1 in MON (B) and STA-WT1 (C) rhabdoid cells. Total proteins were isolated from cells infected with control or INI1-Flag expressing retroviral vectors and subjected to immunoblot analysis. D, Regulation of PLK1 by INI1 in nonrhabdoid tumor cells. HeLa, Jurkats, glioma (SF268), and 293T cells were transfected with either a control siRNA (Si-Control) or siRNA against INI1 (Si-INI1). Total proteins were isolated and subjected to immunoblot analysis to confirm the knock down of INI1. The same immunoblots were then probed to detect PLK1 and tubulin (internal control) proteins. E, Immunohistochemical analysis of human rhabdoid tumors: H&E (left column) and α-PLK1 (right column) staining. The tumor identification is indicated on the left side of each row. BAT, brain adjacent to one of the human rhabdoid tumors. F, Immunohistochemical analysis of mouse rhabdoid tumors: H&E (left column) and α-PLK1 (right column) staining. The tumor identification is indicated on right side of each row. Large bottom panel, high-magnification image of mouse rhabdoid tumor section immunostained with PLK1. Arrow, a rhabdoid cell with eccentric nucleus and enlarged nucleolus (outlined with a dotted line). Note the strong cytoplasmic staining with PLK1 antibody in all rhabdoid cells.
close to 80%, as indicated by immunoblot analysis (Fig. 3A). Down-modulation of PLK1 in rhabdoid cells led to reduction in cell number, increased cell size, and altered cell morphology (data not shown). To determine the effect of down-modulation of PLK1 on mitosis, MON cells transfected with PLK1 siRNA were subjected to immunofluorescence analysis by using α-tubulin antibody (for cytoplasmic staining) and 4',6-diamidino-2-phenylindole (for nuclear staining; Fig. 3B-F). Confocal
microscopic analysis of the cells indicated that a large number of cells transfected with siPLK1 exhibited multiple and fragmented nuclei or aberrant segregation of chromatids (Fig. 3C, E, and F). On the contrary, expression of control siRNA exhibited minimal effect on nuclear morphology and only a few cells expressed aberrant nuclear segregation (Fig. 3C and D). Quantitation of cells with fragmented and multiple nuclei indicated that down-modulation of PLK1 results in a high degree of cells with abnormal nuclei (Fig. 3B). These results indicate that rhabdoid cells are dependent on overexpression of PLK1 for proper mitotic segregation and down-modulation of PLK1 protein leads to abnormal chromosomal/nuclear segregation during mitosis.

**Rapid induction of cell cycle arrest, cell death, and apoptosis in rhabdoid cells upon depletion of PLK1.** To further evaluate the effects of down-modulation of PLK1 in rhabdoid cells, we carried out fluorescence-activated cell sorting analysis. The results indicated that depletion of PLK1 dramatically increased percentage of cells with >2N DNA content (Fig. 4A). Furthermore, the number of cells undergoing apoptosis (sub-G<sub>1</sub> cells) significantly increased in cells treated with PLK1 siRNA compared with the controls (Fig. 4B). To determine if the increase in sub-G<sub>1</sub> population is correlated to a decrease in survival and increase in apoptosis, the rhabdoid cells transfected with siRNAs were replated with equal density and were subjected to survival (MTS) assay 4 days posttransfection. The results indicated that down-modulation of PLK1 inhibited survival compared with that of the controls (Fig. 4C).

Decrease in survival and increase in sub-G<sub>1</sub> cells is indicative of induction of apoptosis in rhabdoid cells. To determine the mechanism of apoptosis mediated by PLK1 inhibition, we assayed the cells transfected with control or PLK1 siRNA for caspase-3/7 activity using a liquid activity assay (CaspaseGlow). We found that there was a significant increase in caspase-3/7 activity in cells treated with PLK1 siRNA ($P < 0.0023$; Fig. 4D). These results showed that down-modulation of PLK1 in rhabdoid cells induce mitotic arrest, caspase-dependent apoptosis, and decreased cell survival. Together, these results suggested that inhibiting expression of PLK1 is deleterious to the survival of rhabdoid cells.

**Discussion**

In this report, we have attempted to identify downstream effectors of INI1/hSNF5 that can be targeted for inhibiting the growth of rhabdoid cells. Although the approach of cDNA microarray analysis to determine INI1-induced gene expression profile changes have been reported earlier (15–17), these studies did not attempt to target downstream effectors of INI1/hSNF5. Our results reveal several novel downstream targets of INI1 such as MMP1 and PLK1 that were not reported earlier. More importantly, we were able to validate the expression pattern of a downstream target gene (PLK1) in primary human and mouse rhabdoid tumors, for the first time, and show the utility of targeting this gene for inhibiting the rhabdoid cell growth.

**Gene expression changes in INI1-induced flat cells.** We found that genes indicative of both differentiation and senescence (MMP1, PAI-1, and extracellular matrix proteins) are upregulated by INI1 at late time points. It is possible that INI1 may play independent roles in both senescence and differentiation. Future experiments to understand these roles of INI1 are likely to shed light on the mechanism of tumor suppression by this gene.

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**Fig. 4.** Effect of down-modulation of PLK1 on growth and survival of rhabdoid cells: A, fluorescence-activated cell sorting analysis of MON cells transfected with PLK1 or control siRNA stained with propidium iodide. B, fold increase in sub-G<sub>1</sub> cell population in MON cells transfected with control or PLK1 siRNA as determined by fluorescence-activated cell sorting analysis in (A). C, percentage of cell survival of MON cells transfected with control or PLK1 siRNAs, as determined by MTS assay. D, fold change in caspase-3/7 activity in MON cells transfected with control or PLK1 siRNAs, as determined by CaspaseGlo caspase-3/7 activity assay.
Induction of IFN signal by INI1 in rhabdoid cells. We have found that INI1 activates IFNs signal in rhabdoid cells during early time points. This was not reported in rhabdoid cells by other studies, perhaps because of the difference in the system used. Based on published studies, it seems that INI1 is required for IFN signaling at two levels. First, it is required for induction of IFNs. Second, it primes the IFN-stimulated gene promoters for rapid induction when IFN signaling occurs. Addition of IFNs partially mimics the effects of INI1 perhaps by increasing the signaling to IFN-stimulated genes. Thus, either the presence of SWI/SNF complex or excess signaling to the promoter could stimulate IFN-stimulated gene expression.

Our results show a link between tumor suppression by INI1 and IFN signaling for the first time. Connection between IFN pathway and other tumor suppressors such as p53 has been reported before (46). It was shown that growth-inhibitory effects of IFN in transformed cells was dependent on p53 and that addition of IFN induces p53 transcription (46). It is possible that there is a link between INI1, IFN, and p53 in inducing growth inhibition of tumor cells. Although INI1 induces IFNs and IFN-stimulated genes, IFNs induce p53, leading to growth arrest. We believe that future experiments to understand the role of IFN signaling by INI1 during tumor suppression may provide further mechanistic insights into its function.

Our results show that IFN treatment results in cell cycle arrest and flat cell formation in rhabdoid cells. IFNs inhibit growth of cancer cells more efficiently than that of the normal cells (46). IFNs are used effectively for the treatment of many cancers (47, 48). Therefore, type I or type II IFN therapy, alone or in combination, is a possible option for the treatment of rhabdoid tumors. However, further in vivo studies will be necessary before taking this concept further into clinic.

INI1 represses mitotic genes. Our results also show that INI1/hSNF5 represses mitotic genes. These studies are consistent with the idea that INI1 mediates mitotic spindle checkpoint control indirectly via p16/cyclin D1/prb/E2F pathway (16) and suggests that perhaps in addition to activating p16, repression of mitotic genes plays a role in this function of INI1. Interestingly, we found that repression of PLK1 by INI1 is specific to rhabdoid tumor cells and is not observed in nonrhabdoid tumor cells. This cell type–specific repression could be due to the presence of cell type–specific repressor components in rhabdoid cells. Future experiments to understand this cell type–specific repression may shed light on the specificity of INI1 to rhabdoid tumor cells.

Our observation suggests that the repression of mitotic genes by INI1 is not a mere consequence of G1 arrest. This is because immunohistochemical analysis of primary rhabdoid tumors lacking INI1 revealed a dramatic overexpression of PLK1 in all tumor cells (Fig. 2E and F). If INI1 has no effect on PLK1 and if its expression was a mere consequence of stage of cell cycle, then only a fraction of tumor cells, which are undergoing G2-M stage, are expected to express PLK1. Instead all the cells of the primary rhabdoid tumors we examined thus far expressed PLK1 arguing strongly for a direct genetic link between INI1 loss and PLK1 overexpression. Our observation also indicates that perhaps PLK1 is an additional diagnostic marker for rhabdoid tumors.

Overexpression of PLK1 protein has been observed in many tumors and is a prognostic marker in some cases (43). Expression of PLK1 seems to be tightly regulated by many tumor suppressors, including Chk1 and BRCA1, to prevent transformation (43). Therefore, it is possible that the PLK1 overexpression due to loss of INI1 may explain poor prognostic nature of rhabdoid tumors.

PLK1 is a potential therapeutic target for rhabdoid tumors. PLK1 is currently an attractive target for developing anticancer therapy (42, 45). It seems that although tumor cells are sensitive to down-modulation of PLK1 due to induction of mitotic catastrophe, normal diploid cells seems to tolerate its inhibition (22, 44). In this report, for the first time, we have shown that down-modulation of PLK1 by RNA interference induces rapid cell cycle arrest, aberrant chromosomal segregation, and apoptosis in rhabdoid cells. Furthermore, we found that down-modulation of PLK1 in other cell types such as 293T was not effective in inhibiting the cell growth, indicating that rhabdoid cells are more sensitive to down-modulation of PLK1 compared with the other cell types (data not shown). These observations support the notion that PLK1 is an objective for developing novel, molecularly targeted therapy for rhabdoid tumors.

Thus far, the chemotherapy regimen used for rhabdoid tumors are empirically based. Our study is an example of using in vitro genetic analysis to identify genes and pathways important for mediating tumor suppression by INI1/hSNF5 and validating their potential as candidates for developing targeted therapy. We propose that single or combination of drugs that induce IFNs or target INI1 downstream genes, such as cyclin D1 and PLK1, could be effective in inhibiting rhabdoid tumors in children.

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References


INI1 Induces Interferon Signaling and Spindle Checkpoint in Rhabdoid Tumors
