

Genetic ablation of *Cyclin D1* abrogates genesis of rhabdoid tumors resulting from *Ini1* loss

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Rhabdoid tumors are aggressive pediatric malignancies for which, currently, there are no effective or standard treatment strategies. Rhabdoid tumors arise because of the loss of the tumor suppressor gene *INI1*. We have previously demonstrated that *INI1* represses *Cyclin D1* transcription in rhabdoid cells by directly recruiting histone deacetylase 1 complex to its promoter, leading to G₀-G₁ arrest. Expression of *Cyclin D1* overcomes cell cycle arrest mediated by *INI1* and *Cyclin D1* overexpression in human rhabdoid tumors is a common phenomenon. However, it is not clear whether *Cyclin D1* is a critical downstream target of *INI1* *in vivo* and whether the derepression of this gene is essential for rhabdoid tumorigenesis. To determine the requirement of *Cyclin D1* for genesis of rhabdoid tumors *in vivo*, we developed *Ini1* heterozygous mice by targeted disruption. We found that the tumors developed in these *Ini1*^{+/-} mice are rhabdoid, defective for *Ini1* protein, and like the human tumors, express *Cyclin D1*. We crossed *Ini1*^{+/-} mice to *Cyclin D1*^{-/-} mice and found that *Ini1*^{+/-} mice with *Cyclin D1* deficiency did not develop any spontaneous tumors, in contrast to the parental *Ini1*^{+/-} mice. These results strongly support the hypothesis that *Cyclin D1* is a key mediator in the genesis of rhabdoid tumors. Our results provide an *in vivo* proof of concept that drugs that target *Cyclin D1* expression or activity could be potentially effective as novel therapeutic agents for rhabdoid tumors.

INI1/hSNF5 | cell cycle | atypical teratoid | tumorigenesis

Rhabdoid tumors, including malignant rhabdoid tumors of the kidneys (MRT) and atypical teratoid and rhabdoid tumors (AT/RT) of the brain and soft tissues, are highly aggressive pediatric malignancies (1–3). Prognosis for infants with these tumors is poor and ≈80% of the children die within 1 yr of diagnosis despite intensive treatment. Chemotherapy is effective only transiently in some AT/RT patients and the use of radiotherapy is limited in children under 3 yr of age because of subsequent neurological damage and because surgical resection is challenging (3). In the past several years, there has been significant progress in the cytogenetic and molecular genetic studies, which indicated that the majority of MRT and AT/RT tumors harbor recurrent biallelic alterations in the *INI1*/hSNF5 gene, located in chromosome 22q11.2 (4–6). Several studies support the model that *INI1* is a tumor suppressor. Children who harbor germ-line constitutive mutation in one allele of *INI1* develop MRT or AT/RT when the second allele of *INI1* is also mutated (5), and families who are carriers of a mutation in this gene are predisposed to rhabdoid syndrome (7). Furthermore, mice heterozygous for *Ini1*/hSNF5 mutations develop rhabdoid tumors with a high frequency because of loss of heterozygosity at the *Ini1* locus (8–10).

INI1/hSNF5 is a component of the chromatin-remodeling mammalian SWI/SNF complex (11). Reintroduction of this gene into rhabdoid cell lines causes G₀-G₁ arrest and affects the transcription of several cell cycle regulatory genes (12). We have demonstrated that *INI1*/hSNF5 represses the transcription of the *Cyclin D1* gene by recruiting the histone deacetylase complex directly to its promoter and that coexpression of *Cyclin D1* from

a heterologous promoter overcomes the cell cycle arrest caused by *INI1* (12). Immunohistochemical analysis demonstrated that *Cyclin D1* is derepressed in human AT/RT tumors that lack functional *INI1* (12). Further analysis indicated that *INI1* also activates p16INK4a (13) and represses *Cyclin E* and *Cyclin A* (12). Furthermore, recent studies suggest that *INI1*/hSNF5 may be involved in activating the mitotic checkpoint through the p16-Cyclin D1/CDK4-pRb-E2F pathway (14).

Cyclin D1 is overexpressed in many human tumors (15–20). However, in the majority of these cases it is not clear whether *Cyclin D1* is actually required for tumorigenesis. Mouse models have demonstrated that *Cyclin D1* function is specifically required for the genesis of a subset (e.g., Ras-Neu-induced breast cancer) but not all cancers (21). *Cyclin D1* is an important cell cycle protein, required to overcome the restriction point in the G₁ stage of the cell cycle (22–24). By binding to cyclin-dependent kinase (cdk) 4/6, *Cyclin D1* facilitates the phosphorylation of Rb to mediate G₁ to S progression. In addition, a cdk-independent function of *Cyclin D1* is involved in modulating the activity of several transcription factors and may be important for tumorigenesis (17, 25–29). It is possible that derepression of *Cyclin D1* due to *INI1* loss in rhabdoid cells is a key event in the genesis of these tumors. Genetic knockout studies in mice indicate that *Cyclin D1* is not essential for survival, probably due to redundancy of D-type cyclins (30). Mice deficient for *Cyclin D1* exhibit a few developmental disorders (30), including reduced body size, reduced viability, and symptoms of minor neurological impairment. In addition, these mice exhibit reduction in retinal cells in the adult, a unique pattern of photoreceptor cell death, and lack of breast epithelium that undergoes proliferative changes during pregnancy (30). Interestingly, the D-type cyclins show specificity during development, and the mice lacking specific D-type cyclins show narrow, restricted developmental abnormalities and distinct phenotypes (30, 31). These results indicate that although the D-type cyclins have similar function during cell cycle, their different cell type distribution and specific roles in development attest to their unique function.

In this report we have tested the *in vivo* requirement of *Cyclin D1* for the genesis of rhabdoid tumors. We generated *Ini1*^{+/-} mice by targeted disruption and found that the tumors developed in these mice are rhabdoid and exhibit derepression of *Cyclin D1*. Furthermore, we crossed these *Ini1*^{+/-} mice to the *Cyclin D1*^{-/-} mice and found that the lack of *Cyclin D1* resulted in abrogation of rhabdoid tumor genesis. These striking results establish a direct link between *INI1* and *Cyclin D1* *in vivo*. Furthermore, these studies provide a proof of principle to indicate that inhibition of *Cyclin D1* or its pathway may be a good therapeutic strategy for treatment of children with rhabdoid tumors.

Abbreviations: AT/RT, atypical teratoid and rhabdoid tumors; ES cells, embryonic stem cells.

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Methods

Generation of Targeting Construct, Gene Targeting, and Germ-Line Transmission. An *Ini1* genomic DNA fragment was isolated by screening the RPCI-22 mouse bacterial artificial chromosome genomic library arrayed on filters, using the exon 7 portion of the mouse *Ini1* cDNA as the probe. A 15-kb genomic DNA fragment was cloned into pBluescript vector, and subsequently the targeting vector was generated by replacing the XbaI to StuI fragment that contains exons 6 and 7 regions with a hygromycin cassette. WW6 embryonic stem cells (32) were electroporated with the targeting construct and selected for hygromycin-resistant colonies as per standard techniques. Pools of 400 clones were screened by long-range PCR for the presence of a 4.2-kb fragment, using primers D149 (5'-GAG CCC AGA AAG CGA AGG-3'), which hybridizes to the hygromycin cassette, and mIEK-3'A (5'-GGG CAA GCT GCT GAT ACA AT-3'), which hybridizes to the 3'-flanking region, outside the targeting sequence. Two different homologous recombinant embryonic stem cell (ES cell) clones were injected into C57BL/6 recipient blastocysts. Male transmitting chimeric mice were obtained and crossed with C57BL/6 females to obtain heterozygous *Ini1*^{+/ Δ exon6,7} F₁ mice. *Ini1*^{+/ Δ exon6,7} mice were crossed with each other to obtain the F₂ generation. For germ-line analysis, DNA from mice was genotyped by PCR using primers specific for the hygromycin cassette: D141, 5'-TGA GCT GGC CCT TAA TTT GG-3', and J65, 5'-TGT GTA GAA GTA CTC GCC GA-3'.

Generation of *Ini1*/Cyclin D1 Double Mutant Mice. *Cyclin D1*-knockout mice (of the C57BL/6J strain) generated by Sicinski *et al.* (30) were a kind gift of R. G. Pestell (Georgetown University School of Medicine, Washington, DC). Two females and two male heterozygous *Cyclin D1*^{+/-} mice were mated with *Ini1*^{+/ Δ exon6,7} mice to obtain *Ini1*^{+/ Δ exon6,7} *Cyclin D1*^{+/-} double heterozygous mice. The double heterozygous mice were mated with littermates to obtain mice with different combinations of genotypes. *Ini1* genotyping was done as described above, and *Cyclin D1* genotyping was done as described in ref. 30.

Cell Culture and RT-PCR Analysis. *INI1*^{-/-} cell lines were grown as described (12). MON cells were transfected with pCGN-*INI1*/hSNF5 expressing HA-*INI1* as described in ref. 12. RNA was isolated from transfected cells and subjected to RT-PCR analysis. Primers used for RT-PCR analysis are as follows: for Cyclin D1, 5'-CTT CCT CTC CAA AAT GCC AG-3' and 5'-AGA GAT GGA AGG GGG AAA GA-3'; for Cyclin D2, 5'-TTC ATT GCA GAC ACC ACC AT-3' and 5'-GCC AGA TAC CAG AAG CGA AG-3'; for Cyclin D3, 5'-TGA TTT CCT GGC CTT CAT TC-3' and 5'-AGC TTG ACT AGC CAC CGA AA-3'; for p16, 5'-CGG AAG GTC CCT CAG ACA TC-3' and 5'-TCA TGA AGT CGA CAG CTT CCG-3'; and for p21, 5'-CTC AGA GGA GGC GCC ATG-3' and 5'-GGG CGG ATT AGG GCT TCC-3'. The PCR was performed in a 25- μ l reaction by using 55°C as the annealing temperature and 1.5 mM MgCl₂ and amplification for 25 cycles. PCR using primers specific to the *GAPDH* gene (5'-GGT GAA GGT CGG AGT CAA CG-3' and 5'-CAA AGT TGT CAT GGA TGA CC-3') was also carried out as an internal control, using the same RNA samples and same conditions.

Protein Expression and Western Blotting. Immunoblotting was performed by standard techniques using antibodies specific for the following proteins: Cyclin D1 (rabbit polyclonal from NeoMarkers, Fremont, CA), Cyclin D2 (rabbit polyclonal, catalog no. sc-181, Santa Cruz Biotechnology), Cyclin D3 (mouse monoclonal, catalog no. c-7214, Sigma), affinity-purified *Ini1* antibodies (33), α -tubulin (catalog no. T5168, Sigma), and β -actin (mouse monoclonal, catalog no. A 5441, Sigma).

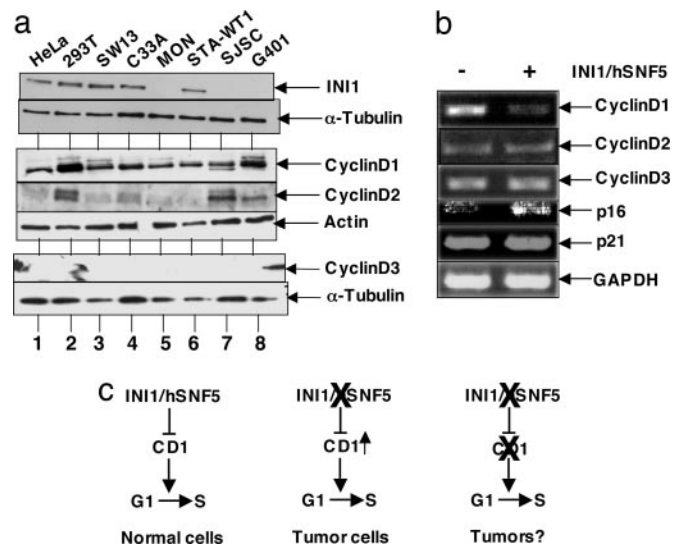


Fig. 1. *INI1* regulates expression of Cyclin D1 but not Cyclin D2 or Cyclin D3. (a) Western analysis of lysates from *INI1*^{+/+} and *INI1*^{-/-} cell lines. Total protein from *INI1*^{+/+} cells (HeLa, lane 1; 293T, lane 2; SW13, lane 3; and C33A, lane 4) and *INI1*^{-/-} cells (MON, lane 5; STA-WT1, lane 6; SJSC, lane 7; and G401, lane 8) were separated by SDS/PAGE and probed with antibodies to *INI1*, α -tubulin, Cyclin D1, Cyclin D2, β -actin, and Cyclin D3. (b) RT-PCR analysis of the RNA isolated from MON cells transfected with pCGN-*INI1*/hSNF5 expressing HA-*INI1* or just pCGN control. (c) Graphic representation of the hypothesis that abrogation of Cyclin D1 (CD1) may result in loss of rhabdoid tumorigenesis.

Immunohistochemical Analysis of Tumor Sections. Immunohistochemistry was performed on mouse tumors developed in *Ini1*^{+/ Δ exon6,7} mice and in *Ini1*^{+/ Δ exon6,7} mice with *Cyclin D1*^{+/+} or *Cyclin D1*^{+/-} background and on a sample of normal brain present adjacent to one of the tumors. Affinity-purified *Ini1* antibodies were used at 1:50 dilutions. For Cyclin D1 immunostaining, a mouse monoclonal antibody, mAbDCS-6 (Cell Marque, Austin, TX) was used at 1:100 dilution. For vimentin (vimentin V9, Ventana Medical Systems, Tucson, AZ) and smooth muscle actin (Dako), we used 1:100 dilutions. For each case, 5- μ m-thick sections were cut from the paraffin blocks, deparaffinized, rehydrated, and stained by standard methods, using the avidin-biotin-horseradish peroxidase complex to localize the antibody bound to antigen, with diaminobenzidine as the final chromogen. Appropriate antigen retrieval was performed in the appropriate buffer. Immunostaining was performed on an automated immunostainer, NexES (Ventana Medical Systems). All immunostained sections were lightly counterstained with hematoxylin. For negative controls, the immune serum was replaced by either PBS or nonimmune serum. The sections were reviewed by the pathologist author (D.Z.).

Results

Expression of Cyclin D1 and Repression of D-Type Cyclins by *INI1* in Rhabdoid Cell Lines. To determine whether the D-type cyclins are specifically overexpressed in rhabdoid tumors as a consequence of loss of *INI1*, we first analyzed the levels of Cyclin D1 in cell lines derived from human rhabdoid tumors and then tested the specificity of regulation of transcription of three D-type cyclins by reintroduction of *INI1* into these cells. We carried out an immunoblot analysis of four different rhabdoid cell lines defective for *INI1*, including STA-WT1, a cell line that carries a cytoplasmically localized, inactive form of the protein (33). We found that Cyclin D1 is the only D-type cyclin that was consistently expressed in all these cell lines (Fig. 1a, lanes 5–8).

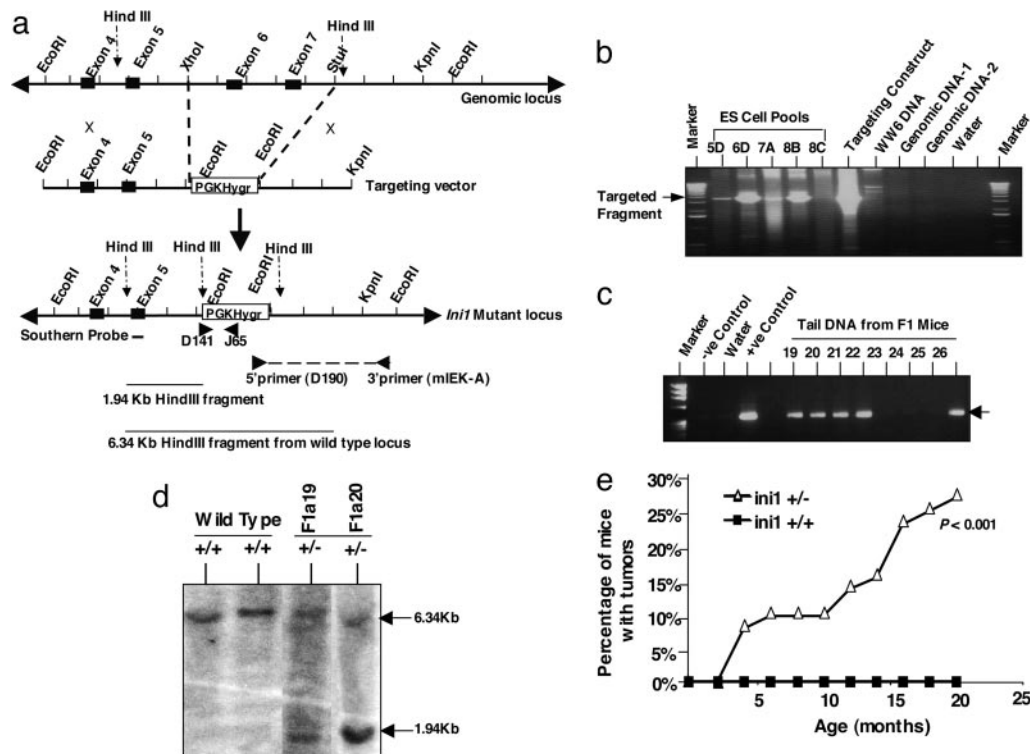


Fig. 2. Targeted disruption of *Ini1* locus and analysis of tumor formation. (a) The mouse *Ini1/Snf5* genomic locus and targeting strategy. (b) Long-range PCR analysis of the pools of ES cell clones to detect the correct targeting. Genomic DNA-1 and -2 are negative controls that harbor the hygromycin cassette in an unrelated locus. The targeting construct was used as a positive control. (c) Germ-line transmission of the correct targeted allele to the F₁ generation was determined by PCR of the tail DNA by using primers (D141 and J65) specific to the pGK-Hygro cassette, which results in the amplification of a 325-bp fragment by PCR. (d) Southern analysis of the DNA isolated from wild-type and heterozygous mice. Correct targeting is indicated by the presence of a 1.9-kb HindIII fragment by using a probe specific to exon 5. (e) Frequency of tumor formation in *Ini1*^{+/*Δ*exon6,7} heterozygous mice.

Although D2 expression was not consistent, D3 was undetectable in all of the cell lines examined, indicating that Cyclin D1 expression is a common feature of these rhabdoid cell lines and tumors (Fig. 1*a*, lanes 5–8). Previously, we demonstrated that INI1 is directly recruited to Cyclin D1 promoter and represses its expression, by using chromatin-immunoprecipitation assays (12). To determine whether the repressive effect of INI1 is restricted to Cyclin D1, we tested the effect of reintroduction of INI1 into the *INI1*^{-/-} MON cell line on the expression of a panel of genes including the three D-type cyclins. RT-PCR analysis of the mRNA expressed in the presence or absence of INI1 indicated that INI1 specifically repressed *Cyclin D1* but not *D2* and *D3* (Fig. 1*b*). Furthermore, as reported in ref. 13, INI1 also activated *p16*^{INK4a} but had no effect on *p21*^{Kip1} expression (Fig. 1*b*). These results indicate the specificity of repression of *Cyclin D1* by INI1 in MON cells.

Cyclin D1 Is Derepressed in Mouse Rhabdoid Tumor Models. Based on the observations that (i) *Cyclin D1* but not *D2* and *D3* is specifically repressed by INI1 in rhabdoid cells; (ii) expression of *Cyclin D1* from a heterologous promoter is sufficient to overcome the INI1-mediated cell cycle arrest (12); and (iii) Cyclin D1 is expressed in human rhabdoid cell lines and tumors (12), we surmised that Cyclin D1 is a key downstream target of INI1-mediated tumor suppression. We further hypothesized that rhabdoid tumors are critically dependent on *Cyclin D1* expression for survival and that ablating *Cyclin D1* would result in the lack of or at least reduced frequency of tumors that arise because of the loss of the *Ini1* gene (Fig. 1*c*). To test this hypothesis, we first generated mice carrying heterozygous mutations in *Ini1* by targeted disruption. The genomic *Ini1* exons 6 and 7, which

encode the highly conserved repeats 1 and 2, were replaced with a hygromycin cassette to generate the mutant allele *Ini1*^{Δexon6,7} (Fig. 2*a*). Correct targeting of the *Ini1* locus was identified first by screening the pools of ES cell clones and then by screening the individual clones by long-range PCR using a primer pair, one specific for the hygromycin cassette and another spanning the 3'-flanking sequence adjacent to the targeted region (Fig. 2*a* and *b*). These analyses indicated the presence of homologous recombination in ≈25% of ES cell clones. The positive ES cells were microinjected into blastocysts, and germ-line transmission was monitored by PCR analysis using primers specific to the hygromycin cassette (Fig. 2*c*). Correct targeting was confirmed by Southern analysis of mouse-tail DNA obtained from F₁ mice (Fig. 2*d*).

Analysis of the F₁ and F₂ generations indicated the lack of live-born mice homozygous for the *Ini1*^{Δexon6,7} allele, consistent with earlier reports that the *Ini1*-null genotype is embryonic lethal (8, 10, 34). Immunoblot analysis of total protein isolated from heterozygous mice with an anti-*INI1* antibody that recognizes the N-terminal domain demonstrated the presence of only the full-length *Ini1* protein (but no truncated protein), indicating that *Ini1*^{Δexon6,7} is likely a null allele (data not shown). *Ini1*^{+/*Δ*exon6,7} heterozygous mice were obtained in a normal Mendelian ratio and were devoid of any apparent developmental or behavioral disorders. Observation of the cohort of mice for nearly 2 yr revealed the development of spontaneous face and brain tumors, at a very high frequency (≈26%) (Figs. 2*e* and 3*A*), and a small percentage of mice developed tumors in the hind limb. The tumors in *Ini1*^{+/*Δ*exon6,7} mice developed with an early onset of 3 months and a median onset of ≈11 months of age. They were histopathologically

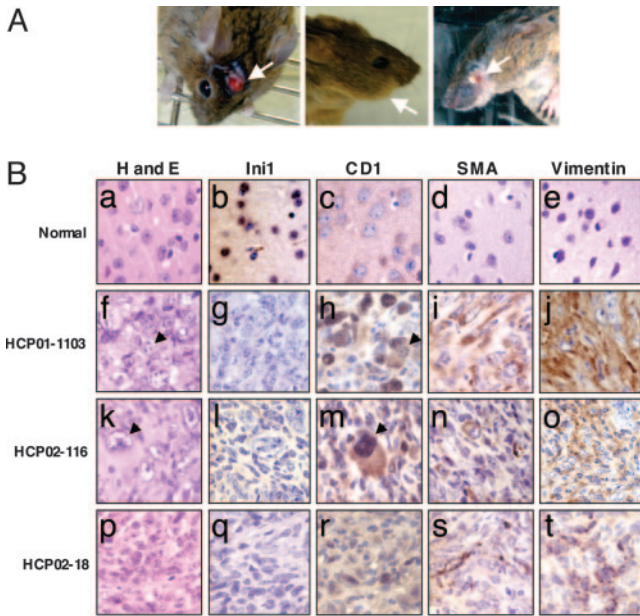


Fig. 3. Cyclin D1 is overexpressed in tumors developed in *Ini1*^{+/ Δ exon6,7} mice. (A) *Ini1*^{+/ Δ exon6,7} mice develop head and face tumors. (Left) Tumor present in the soft tissue of the brain penetrating the skull. (Center) Enlarged side of the face because of the presence of tumor mass behind the cheek. (Right) Tumor under the cerebellum has dislodged the eye. (B) Immunostain analysis of tumors developed in *Ini1*^{+/ Δ exon6,7} mice. First row: paraffin section of normal brain tissue adjacent to tumor HCP01-1103. Second, third, and fourth rows represent the immunostain analysis of the paraffin sections of three different mouse tumors (HCP01-1103, HCP02-116, and HCP02-18, respectively) developed in *Ini1*^{+/ Δ exon6,7} mice. Column 1, hematoxylin and eosin (H and E) staining (a, f, k, and p). Columns 2–5 represent immunohistochemical staining using anti-*INI1* (b, g, l, and q), anti-Cyclin D1 (c, h, m, and r), anti-smooth muscle actin (SMA) (d, i, n, and s); and anti-vimentin (Vim) (e, j, o, and t) antibodies, respectively. The arrows point to rhabdoid cells.

classified as primary brain tumors, undifferentiated sarcomas, ganglioneuromas, and neuroblastomas, but a closer examination revealed the presence of rhabdoid cells in the majority of these tumors (Fig. 3B). These results of development of rhabdoid tumors in *Ini1*^{+/ Δ exon6,7} heterozygous mice confirm the published work of others (8–10).

A prerequisite to determining the importance of Cyclin D1 in the genesis of rhabdoid tumors is to demonstrate that this gene indeed is derepressed even in mouse rhabdoid tumors. Therefore, we next characterized the mouse tumors by immunohistochemical analysis using affinity-purified anti-*INI1* and anti-Cyclin D1 antibodies, as well as other antibodies against markers characteristic of rhabdoid tumors, such as vimentin and smooth muscle actin (3) (Fig. 3B). The results of these analyses indicated that the mouse tumors are similar to those of human rhabdoid tumors in all respects: They were negative for *Ini1* and positive for vimentin and smooth muscle actin. Lack of *Ini1* in the tumor tissue is consistent with the fact that these tumors arose because of the loss of heterozygosity at the *Ini1* locus. Importantly, Cyclin D1 was well expressed in all of the tumors examined, including the rhabdoid cells present in these tumors (Fig. 3B). These results are consistent with our hypothesis that *Ini1* loss leads to the derepression of *Cyclin D1*. Thus, these knockout mice represent an excellent model system for testing the dependence on Cyclin D1 for genesis of rhabdoid tumors.

Cyclin D1 Is Necessary for Rhabdoid Tumor Genesis. Because *INI1* represses Cyclin D1 and because Cyclin D1 is derepressed in rhabdoid tumors, it is possible that expression of Cyclin D1 is a

prerequisite for the genesis of rhabdoid tumors. To test the hypothesis that Cyclin D1 derepression is necessary for the rhabdoid tumors formation in mice, we crossed the heterozygous *Ini1*^{+/ Δ exon6,7} mice with *Cyclin D1*^{+/-} mice to obtain double heterozygous mice. Mice carrying targeted alleles of *Cyclin D1* were generated by Sicinski *et al.* (30) by replacing one-half of exon 1 and all of exons 2 and 3 with a neomycin cassette. *Ini1* and *Cyclin D1* heterozygous mice that were obtained from these crosses were mated to each other to generate *Ini1*^{+/ Δ exon6,7} mice with three different *Cyclin D1* backgrounds (*Cyclin D1*^{+/+}, *Cyclin D1*^{+/-}, and *Cyclin D1*^{-/-}). No mice homozygous for *Ini1* mutation were found in these crosses, indicating that *Cyclin D1* mutation is not epistatic for the embryonic lethality caused by homozygous *Ini1* deletions. We obtained two cohorts of mice of six different genetic backgrounds (*Ini1*^{+/+} or *Ini1*^{+/-} mice with three different *Cyclin D1* genotypes) and observed them for a period of 15 months. We found that the *Ini1*^{+/+} mice with three different *Cyclin D1* genotypes exhibited expected phenotypic characteristics as reported earlier (30). Whereas the *Cyclin D1*^{+/+} and *Cyclin D1*^{+/-} mice exhibited normal survival rates, the *Cyclin D1*^{-/-} mice demonstrated reduced rate of survival because of early neonatal mortality (Fig. 4A). The *Cyclin D1*^{-/-} mice were smaller than their littermates and exhibited minor neurological and retinal developmental defects in both *Ini1*^{+/+} and *Ini1*^{+/-} backgrounds, indicating that these characteristics are due to the manifestation of the *Cyclin D1* lesions and that they were not related to *Ini1* heterozygosity (Fig. 4).

Comparative analysis of survival of the cohort of *Cyclin D1* mice with two different *Ini1* genetic backgrounds is depicted in Fig. 4 B–D. We found that both *Cyclin D1*^{+/+} and *Cyclin D1*^{+/-} mice with the *Ini1*^{+/-} heterozygous background exhibited a reduced survival rate as compared to the *Ini1*^{+/+} mice with similar *Cyclin D1* background (Fig. 4 B and C). However, there was no significant difference in the survival rate of *Cyclin D1*^{-/-} mice with either *Ini1*^{+/+} or *Ini1*^{+/-} background. Both of these mice exhibited early neonatal mortality, but adult mice survived at an equal frequency (Fig. 4D). We next observed *Ini1*^{+/-} mice with three different *Cyclin D1* genetic backgrounds for tumor formation frequency for a period of up to 15 months. The *Cyclin D1*^{+/+} or *Cyclin D1*^{+/-} mice developed tumors with a frequency similar to what we observed before (26.1% in *Cyclin D1*^{+/+} and 25% in *Cyclin D1*^{+/-}), with a median onset of \approx 9 months of age. The majority of the tumors developed in the face and head. Immunohistopathological characterization of representative tumors from these cohorts of mice indicated that they were rhabdoid in nature. The tumors exhibited the presence of rhabdoid cells with typical morphology, were positive for vimentin and smooth muscle actin, and overexpressed Cyclin D1 (Fig. 4F and data not shown). Interestingly, although one of the tumors we observed was negative for *Ini1* staining, another tumor exhibited strong cytoplasmic localization of the *Ini1* protein (Fig. 4F). This observation strengthens our previous report that in human tumors, one mechanism of inactivation of *INI1* is by mislocalization of the protein in the cytoplasm due to the activation of the masked nuclear export signal (33).

The striking result of these analyses was that none of the *Ini1*^{+/-} mice that carried the *Cyclin D1*^{-/-} genotype developed tumors within the observation period (Fig. 4E). These results are significant ($P < 0.05$) and indicate that *Cyclin D1* deletion overcomes the tumorigenic effect of *Ini1* loss. This observation is in contrast to the effect of *Cyclin D1* deletion on embryonic lethality of *Ini1*^{-/-} mutation and indicates that tumorigenesis due to *Ini1* loss *in vivo* is exquisitely dependent on the presence of *Cyclin D1*.

Discussion

Our results provide strong *in vivo* evidence to support the hypothesis that Cyclin D1 expression is essential for the genesis

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