Sequestration of E12/E47 and suppression of p27^KIP1 play a role in Id2-induced proliferation and tumorigenesis

Valerie A. Trabosh, Kyle A. Divito, Baltazar D. Aguda, Cynthia M. Simbulan-Rosenthal and Dean S. Rosenthal

Department of Biochemistry and Molecular Biology, Georgetown University School of Medicine, 3900 Reservoir Road NW, Washington, DC 20007, USA and 1Mathematical Biosciences Institute, Ohio State University, Columbus, OH 43210, USA

Id2 is a member of the helix-loop-helix (HLH) family of transcription regulators known to antagonize basic HLH transcription factors and proteins of the retinoblastoma tumor suppressor family and is implicated in the regulation of proliferation, differentiation, apoptosis and carcinogenesis. To investigate its proposed role in tumorigenesis, Id2 or deletion mutants were re-expressed in Id2-/- dermal fibroblasts. Ectopic expression of Id2 or mutants containing the central HLH domain increased S-phase cells, cell proliferation in low and normal serum and induced tumorigenesis when grafted or subcutaneously injected into athymic mice. Similar to their downregulation in human tumors, the expression of cyclin-dependent kinase inhibitors p27^KIP1 and p15^INK4b was decreased by Id2; the former by downregulation of its promoter by expression of p27^KIP1 in Id2-overexpressing cells reverted the hyperproliferative and tumorigenic phenotype, whereas deletion of p27 blocked Id2-induced proliferation, suggesting that Id2 mediates its effects primarily by regulating p27^KIP1. These results confirm the role for Id2 as a regulator of the cell cycle with oncogenic properties.

Introduction

Inhibitor of differentiation (Id) proteins comprise a class of helix-loop-helix (HLH) transcription factors unable to bind DNA. Like all seven classes of HLH transcription factors (1,2), the Id HLH motif allows Ids to form heterodimers with the ubiquitously expressed class I HLH (E proteins), and some of their class II HLH tissue-specific protein partners such as MyoD (3). Whereas other HLH classes have I HLH (E proteins), and some of their class II HLH tissue-specific factors and proteins of the retinoblastoma tumor suppressor family and is implicated in the regulation of proliferation, differentiation, apoptosis and carcinogenesis. To investigate its proposed role in tumorigenesis, Id2 or deletion mutants were re-expressed in Id2-/- dermal fibroblasts. Ectopic expression of Id2 or mutants containing the central HLH domain increased S-phase cells, cell proliferation in low and normal serum and induced tumorigenesis when grafted or subcutaneously injected into athymic mice. Similar to their downregulation in human tumors, the expression of cyclin-dependent kinase inhibitors p27^KIP1 and p15^INK4b was decreased by Id2; the former by downregulation of its promoter by the Id2 HLH domain-mediated sequestration of E12/E47. Re-expression of p27^KIP1 in Id2-overexpressing cells reverted the hyperproliferative and tumorigenic phenotype, implicating Id2 as an oncogene working through p27^KIP1. These results tie together the previously observed misregulation of Id2 with a novel mechanism for tumorigenesis.

Materials and methods

Cell culture

Id2-/- neonatal mouse skins were floated on 0.25% trypsin-ethylenediamine-tetraacetic acid (Gibco, Carlsbad, CA) in 4°C, demarcises separated, immersed in 0.35% collagenase and centrifuged at 1200 r.p.m. for 3 min and at 400 r.p.m. twice for 5 min (to eliminate hair follicles). Cells were maintained by standard M199/10% FCS protocol in Dulbecco’s modified Eagle’s medium/penicillin (100 U/ml)/streptomycin (100 μg/ml) supplemented with 10% fetal bovine serum.

Plasmids and recombinant viruses

The Id2 coding region was amplified with primers incorporating Hind III and Cla I sites and ligated into pLH CX. This retroviral construct or empty vector pLH CX was transfected into dNX-packaging cells; all transfections utilized Lipofectamine-LTX (Invitrogen, Carlsbad, CA). Viral supernatants were used to transduce murine Id2-/- dermal fibroblasts. After hygromycin selection (100 μg/ml) for 10 days, cells were passaged every 3 days. Immunoblotting confirmed Id2 expression. All subsequent experiments were performed three times using the same pooled clones. Additionally, growth curves and tumorigenesis assays were performed using pooled clones from a second transduction of each retroviral construct to verify the results of the first transduction.

Mutant constructs were designed using the same method with the following primers:

1. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
2. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
3. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
4. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
5. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
6. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
7. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
8. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
9. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
10. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
11. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
12. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
13. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
14. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
15. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
16. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
17. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
18. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
19. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
20. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';
21. 5' - AGGCTTTAGAAGCTGATCAGTCAATGAC-3' and 3' - ATCGTGACACCGCAACTTGCCGCT-5';

Abbreviations: BrdU, bromodeoxyuridine; EMSA, electrophoretic mobility shift assay; FACS, fluorescence-activated cell sorting; HLH, helix-loop-helix; Id, inhibitor of differentiation; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; Rb, retinoblastoma; RT, reverse transcription; UVB, ultraviolet B.

Using Id2-/- cells as recipients for Id2 and Id2 mutants, we examined the role of Id2 in tumor formation and cell proliferation. Cells expressing Id2 exhibited higher rates of proliferation and showed a pronounced ability to form tumors when grafted or injected into athymic mice, compared with control Id2-/- dermal fibroblasts. Id2 and Id2 mutants containing the HLH domain downregulated endogenous p27^KIP1 RNA and protein levels, as well as the p27^KIP1 promoter via sequestration of E12/E47. Re-expression of p27^KIP1 in Id2-overexpressing cells reverted the hyperproliferative and tumorigenic phenotype, whereas deletion of p27 blocked Id2-induced proliferation, suggesting that Id2 mediates its effects primarily by regulating p27^KIP1. These results confirm the role for Id2 as a regulator of the cell cycle with oncogenic properties.

Proliferation assay

A total of 2 × 10^5 cells were seeded in 100 mm plates, medium changed every 3 days, viability assessed by trypan blue exclusion another day for 14 days. For fluorescence-activated cell sorting (FACS), nuclei were stained with propidium iodide and analyzed with a Becton-Dickinson FACStar Plus (22).

For bromodeoxyuridine (BrdU) staining, cells were pulsed for 2 h with 10 μM bromodeoxyuridine and washed with phosphate-buffered saline (PBS). Cells were fixed with 100% methanol and DNA denatured with 0.07 M NaOH.
in 70% ethanol; cells were washed with PBS and incubated with anti-BrdU (Molecular Probes, Carlsbad, CA; 1:1000) in PBS with 10% goat serum. Cells were washed in PBS and incubated with Alexa Fluor 488-labeled anti-mouse (Molecular Probes, 1:500) and rewarshed. BrdU-positive cells were counted in 10 random fields with ~30 cells per field using a Zeiss immunofluorescence microscope.

Focus assays
Cells were grown past confluence for 2 weeks, with medium changed every 3 days and then stained with crystal violet. For density limitation of growth assays, 1 × 10^5 cells were plated, fed every 3 days and viable cells counted on indicated days.

Reverse transcription–polymerase chain reaction
Reverse transcription (RT)–polymerase chain reaction (PCR) was performed with M-MLV RT (200 U) from Transcend RNA (1 μg), purified from cells with Trizol (Gibco BRL, Carlsbad, CA), utilizing an Applied Biosystems (Foster City, CA), GeneAmp EZ 10 Th RNA PCR kit. After RT at 65°C for 40 min, complementary DNA was amplified by an initial incubation at 95°C for 2 min, followed by 40 cycles of 95°C for 1 min, 60°C for 1.5 min and 65°C for 0.5 min and a final extension at 70°C for 22 min. PCR products were separated by agarose gel electrophoresis.

Promoter assays
A total of 80% confluent cells were cotransfected with pRLTK Renilla vector and a 2 kb p27KIP1 promoter fragment linked to firefly luciferase (20:1 molar ratio using 2 μg DNA per well). For Id2 studies, cells were additionally cotransfected with the retroviral construct pLHCX/Id2 (or mutants) or empty pLHCX in a 1:1 molar ratio of p27KIP1 promoter–luciferase construct: pLHCX/Id2. Twenty-four hours later, luciferase and Renilla activities were measured (Dual Luciferase Reporter Assay System, Promega, Madison, WI).

Electrophoretic mobility shift assay
Nuclear extracts were isolated using NE-PER nuclear and cytoplasmic extraction reagent (Pierce, Rockford, IL). Electrophoretic mobility shift assay (EMSA) was performed using the Lightshift Chemiluminescent EMSA kit (Pierce). Biotinylated oligonucleotide probes containing each of the 5 E-boxes in the p27KIP1 promoters were annealed with each of their reverse complements: E-box #1: 5'-GACGAGTGTGTCACGTTGCACTGACACA3'. E-box #2: 5'-GACGAGTGTGTGTCACGTTGCACTACACA3'. E-box #3: 5'-CAACTCCGCTTCTATCATATGATTCTAGGAAACTG-3'. E-box #4: 5'-AATCTGTATTTGCTAGCTGAACGATAGATAG-3' and E-box #5: 5'-CAGGGTTGTTGAGACGTGACACTTGAAG-3'. Cells (10 μg protein) were preincubated with excess poly(dI:dC) (1 μg) in DNA-binding buffer (Pierce), 2.5% glycerol, 5 mM MgCl₂, 0.05% NP40, in the presence or absence of 1 μg anti-E12/E47 for 20 min. Binding reactions (20 μl) were incubated for 5 min with excess non-biotinylated probes (4 pmol per reaction; for competition assays) and an additional 20 min with biotinylated probes (40 fmol per binding reaction). DNA–protein complexes were resolved on native 5% polyacrylamide gels in Tris–borate–ethylenediaminetetraacetic acid buffer, followed by transfer to nylon membranes. UV cross-linking of DNA to the membrane and detection of biotin-labeled DNA by chemiluminescence.

Chromatin immunoprecipitation
Cells were grown to confluence, fixed in 1% formaldehyde for 10 min, neutralized with 0.125 M glycine, washed in PBS and lysed in 1% sodium dodecyl sulfate, 10 mM ethylenediaminetetraacetic acid and 50 mM Tris–HCl pH 8.0. Chromatin immunoprecipitation (ChIP) was performed according to standard procedures. Immunoprecipitated DNA was purified with antibodies specific to Id2 (C-20), Id1 (C-20), Id3 (C-20), p16INK4a, p18INK4c, p19INK4a, p19INK4d, p21CIP1, p27KIP1, p57KIP2, cyclin D1, cdk4, retinoblastoma (Rb) and E47 (Santa Cruz; 1:200) or to glyceraldehyde 3-phosphate dehydrogenase for loading control (1:4000; Chemicon, Billerica, MA). Immune complexes were detected by horseradish peroxidase-conjugated antibodies (1:5000) and enhanced chemiluminescence (Pierce).

Grafting protocols and tumor xenografts
A 1 cm diameter piece of skin was removed from the dorsal surface of athymic mice, and 8 × 10^6 fibroblasts + 5 × 10^9 primary human keratinocytes were pipetted on top of the muscular layer within a silicon dome. The dome was removed after a week and the skin graft was allowed to develop for 6 weeks. For subcutaneous injection, athymic mice were injected subcutaneously with 9 × 10^6 cells with a 25 ga needle in the hindquarter. Mice were monitored for signs of tumor growth and tumor length, width and height were measured biweekly using a caliper. Each testing group contained at least three mice in one experiment. The results were expressed as the mean (±SE) of tumor volume in each group. After 5 weeks, mice were killed, tumors were surgically removed and used for histology and imaging.

Statistical analysis
Data from three experiments were compared using Student’s t-test for significance and P values of <0.05 were considered statistically significant. The results are representative of three independent experiments with reproducible results.

Results
Re-expression of Id2 induces increased cell proliferation in Id2+/− fibroblasts
Id2-null, heterozygotic and wild-type neonates were assayed by PCR for Id2 and/or neomycin genes (23). DNA from Id2+/− mice yields a single-Id2 band, whereas DNA from Id2−/− mice exhibits one band representing neomycin that replaced the first two exons of Id2; DNA isolated from heterozygous Id2+/− mice reveals two bands (Figure 1A, left). Dermal fibroblasts were isolated from the skin of homozygous Id2−/−, and a retroviral suspension was used to transduce Id2−/− cells with either Id2/LHCX or LHCX alone. Following a 10 day selection with hygromycin, RNA and protein were isolated from the stable cell lines of cloned polones. RT–PCR showed re-expression of Id2 RNA in Id2−/− cells following transduction (Figure 1A, middle). Immunoblot analysis shows that Id2/LHCX cells containing the stably integrated retroviral vector express Id2 protein (Figure 1A, right).

Cell lines were plated at equal density and cells were counted on indicated days for 14 days; cell viability remained at 100% throughout the time course. Id2−/− cells re-expressing Id2 (Id2+/LHCX) have a significantly higher growth rate compared with Id2−/− cells stably transduced with LHCX empty vector (Figure 1B, left). FACS revealed a significantly higher percentage of Id2/LHCX cells in S phase at all time points (Figure 1B, right). Cells were pulse labeled and stained with fluorescein-conjugated anti-BrdU. BrdU-positive cell numbers were significantly increased in Id2/LHCX cells (Figure 1C). Serum was withdrawn from 10 to 1% and cells counted on days 0, 2 and 6. Figure 1D shows a significantly greater number of Id2/LHCX cells on days 2 and 6, indicating a low-serum growth advantage conferred by Id2.

Re-expression of Id2 induces neoplastic transformation in Id2−/− fibroblasts
The effect of Id2 expression on focus formation, a classical indicator of neoplastic transformation, was next examined. Representative images of plates of cells allowed to grow past confluence and form foci are shown and quantified in Figure 2A. Ectopic expression of Id2 in Id2/LHCX cells decreased contact inhibition of cell growth as shown by increased numbers and sizes of foci, indicating transformed cells.

To determine the effects of Id2 on density limitation of growth, 1 × 10^5 cells were plated and counted on the days indicated in Figure 2B. The saturation density for cells expressing Id2 was significantly higher than control cells, further indicating a loss of contact inhibition.

Re-expression of Id2 causes tumor formation in athymic mice
Proliferation (Figure 1), focus-forming (Figure 2A) and soft agar assays (not shown) indicated that Id2-overexpressing cells, but not Id2-null cells, may be tumorigenic in vivo. A grafting protocol (24,25) was used to test the tumor-inducing capability of Id2. Stable dermal fibroblasts were combined with primary human keratinocytes and grafted onto the dorsum of athymic mice. Id2-overexpressing...
fibroblasts formed tumors, whereas vector control fibroblasts were unable to induce tumors in the graft system (Figure 2C). LHCX graft sections displayed normal skin histology, whereas an undifferentiated mass of cells was seen in tumors from Id2-expressing grafts suggesting a fibrosarcoma-like tumor. Dermal fibroblasts stably overexpressing Id2 were also subcutaneously injected into the hindquarters of mice and tumor formation was measured over a period of 5 weeks. Figure 2D shows that the same tumorigenic effect of Id2 was observed in this system; the potency of Id2 as a promoter of tumorigenesis was evident when palpable tumors began to form in as little as 1 week postinjection, whereas no tumors formed in the time frame of this study following injection of LHCX cells. Growth curves and tumorigenesis assays were performed using pooled clones from a second transduction of each retroviral construct and the results were essentially the same as those obtained in three independent experiments. Technical replicates (e.g. multiple cell counts) were also performed for each of the biological replicates but were not incorporated in the statistics for the three independent experiments.

Id2 controls G₁–S transition via transcriptional downregulation of p27KIP1

Id2 is regulated throughout the cell cycle, with highest expression levels at the G₁-S transition. Since Id2 cannot bind DNA, and given previously noted potentially functional interactions between Id genes and CDKIs (14,15), we determined if Id2 could exert its effects by regulating cell cycle inhibitors. We examined expression of cell cycle regulators of G₁–S transition in the INK and CIP/KIP families, including p21cip1, p27kip1, p57kip2, p16ink4a, p15ink4b, p18ink4c and p19ink4d. We also examined the levels of cyclin D1, cdk4 and Rb, involved in cell cycle progression and tumorigenesis, since Rb has been implicated in sequestering Id2 (26). Figure 3A shows that Id2 strongly decreased levels of p27kip1 and p15ink4a. However, there was no marked change in the levels of other CDKIs, cyclin D1, cdk4 or Rb, suggesting that Id2 may be exerting its effect on cell proliferation largely through p27kip1 and/or p15ink4a downregulation. Since Id1 and Id3 have been shown to play key roles in p27kip1 regulation (27), we also determined whether Id1 or Id3 is affected under these conditions, which could suggest a potential role in mediating the observed Id2 responses. Immunoblot analysis revealed no significant changes in the levels of Id1 or Id3; thus, they do not seem to play an obvious role in this system.

To determine if the regulation of p27kip1 by Id2 was occurring at the RNA level, RNA was isolated from LHCX or Id2/LHCX fibroblasts and subjected to RT–PCR analysis. Figure 3B shows that, similar to their corresponding proteins, the pattern of expression for Id2 is inversely...
related to the expression of p27KIP1 RNA, indicating that Id2 suppresses
the levels of p27KIP1 at the RNA transcriptional or posttranscriptional
level. Consistent with the results of immunoblot analysis, Id1 and Id3
RNA levels were also unaffected by Id2 expression.

Immunoblot analysis of p27 KIP1 protein levels under different
serum conditions was also performed to determine whether
p27KIP1 expression differences resulting from Id2 overexpression
confers a proliferative advantage in low serum (shown in Figure
1D), as well as the ability to grow to higher densities (Figure 2B).
At all time points, cell viability was 100% (data not shown). As expected, p27 KIP1 expression in vector-
transduced cells was slightly increased by low-serum levels, which
is consistent with the fact that p27KIP1 expression can be upregulated
by mitogen deprivation [Figure 3C, top; (28)]. More significantly,
p27 KIP1 expression was completely abolished by Id2 overexpression
in either high or low serum. Furthermore, p27KIP1 was induced by
high density in LHCX but not in Id2-overexpressing cells over an
11 day period (Figure 3C, bottom).

To determine if repression of p27KIP1 by Id2 was exerted at the
transcriptional level, a 2 kb fragment of the p27KIP1 promoter attached
to a luciferase reporter gene was transfected into cells stably expressing
Id2/LHCX or LHCX empty vector. Id2 significantly decreased
p27KIP1 promoter activity suggesting transcriptional control of
p27KIP1 (Figure 3D, top left).

EMSA revealed that E-box #4 binds to E12/E47 in extracts of LHCX,
but not Id2/LHCX cells (Figure 3D, top right). None of the other four E-
boxes formed a stable complex with E12/E47 (data not shown). Chro-
matin immunoprecipitation analysis also shows that E12/E47 binds to
the p27KIP1 promoter in control LHCX cells, and this binding is reduced
in cells stably expressing Id2/LHCX, suggesting that Id2 sequesters
E12/E47 from this promoter (Figure 3D, bottom left), since the total
intracellular levels of E12/E47 are unaltered by Id2 (data not shown).

Id2 mutants
To further elucidate the mechanism by which Id2 regulates p27KIP1
and tumorigenesis, we derived deletion constructs of Id2. Figure 4A
shows the three domains that comprise Id2: the N-terminal domain,
the HLH domain and the C-terminal domain, containing a nuclear
export signal. These regions were cloned into LHCX and packaged
as a retrovirus. The mutants include deletions of either 37 or 78
amino acids from the N-terminus (ΔN37 and ΔN78) or 58 or 97 amino acids from the C-terminus (ΔC58 and ΔC97). The smaller deletions (ΔN37 and ΔC58) eliminate only the N- or C-terminus, whereas the larger deletions eliminate the HLH domain as well.

Id2+/C0+/C0 cells were then used as recipients for viral transduction of each of the four mutants, as well as LHCX vector alone, or full-length Id2. RNA was isolated from each cell line and specific primers demonstrated robust expression of each of the mutants and full-length Id2, whereas immunoprecipitation revealed expression of the corresponding proteins (Figure 4B). The effect of each domain of Id2 on the cell cycle was then examined by FACS as shown in Figure 4C. Full-length Id2, as well as the two mutants retaining the HLH domain (ΔN37 and ΔC58), induced an increase in S phase, whereas LHCX as well as mutants lacking the HLH domain did not. To determine the effects of each domain on tumorigenesis, athymic mice were injected subcutaneously with Id2−/− fibroblast cell lines expressing each of the four mutants, as well as with cells expressing full-length Id2, or with vector alone. Cells expressing Id2, as well as ΔC58, were formed significantly larger subcutaneous tumors than those expressing Id2 mutants lacking the HLH (Figure 4D). ΔN37 formed only small tumors, suggesting an additional role for the N-terminal domain of Id2 for tumorigenesis in vivo.
p27KIP1 mediates the increase in S phase induced by Id2

To determine the contribution of p27KIP1 suppression to the proliferative effects of Id2, control or Id2-expressing fibroblasts were transiently transfected with either Id2 or p27KIP1. Transient transfection followed by RT–PCR revealed a robust expression of either Id2 or p27KIP1 (Figure 5A). Transfected cells were then subjected to FACS analysis to determine the percentages of cells in S phase following expression of p27KIP1 and/or Id2. Figure 5B shows that Id2/LHCX cells transiently transfected with p27KIP1 have a reduced percentage of cells in S phase (lane 2 versus lane 1). When LHCX cells were transfected with p27KIP1, there was no significant reduction in S phase, compared with LHCX alone, and additionally, S phase was not reduced below the level of Id2/LHCX cells transfected with p27KIP1 (lane 3 versus lane 1), suggesting that expression of p27KIP1 is necessary and sufficient to decrease the S-phase population, regardless of Id2 status. This supports the idea that p27KIP1 is mediating the S-phase effects of Id2. As a control, LHCX cells transiently transfected with Id2 strongly decreased p27KIP1 levels (Figure 5A, lane 4) and significantly elevated the S-phase population (Figure 5B, lane 4).

The HLH domain of Id2 is required for suppression of the p27KIP1 promoter

Given the ability of Id2 to suppress p27KIP1 and increase S phase, we determined which region of Id2 was responsible for these effects. Since all our experiments above were carried out in mouse Id2−/− fibroblasts as the parental cells, we also wished to determine whether these results...
Id2 and Id3 are upregulated by UVB exposure, at the messenger RNA and protein levels, in primary and immortalized keratinocytes, respectively (16,29). Id3 induces Bax-dependent apoptosis and mediates UVB sensitization of immortalized human keratinocytes (30), whereas UVB upregulates Id2 in primary human keratinocytes at the promoter level and mediates UVB inhibition of keratinocyte differentiation (16). Id2 has also been shown to be a key player in skin development by maintaining the integrity of vital signaling pathways as well as the proper ratio of each cell type within the skin (23,31–33). Whereas primary mouse dermal fibroblasts were successfully derived from neonatal Id2−/− mice, primary mouse keratinocytes were unable to attach or grow in culture. As an alternative to keratinocytes, dermal fibroblasts were therefore used to examine the effects of ectopic expression of Id2 since Id2 is known to be dysregulated in tumors of fibroblastic origin (18,20). In the current study, we expressed full-length Id2 as well as deletion mutants in Id2−/− dermal fibroblasts to determine their effects on proliferation and tumorigenesis. Id2 dramatically increased the proliferation of these cells. Over 8 days, Id2-expressing cells were able to continue through approximately six population doublings, whereas those cells not expressing Id2 were only able to continue through four population doublings (Figure 1B). Consistent with BrdU analysis (Figure 1C), there were significantly more cells in S phase using flow cytometry (Figure 1B).

To determine if changes in proliferation and focus formation (Figure 2) in vitro would reflect an in vivo model, cells were grafted or subcutaneously injected into nude mice. No tumors were formed by Id2−/− cells, whereas substantial tumors were evident on animals injected with cells ectopically expressing Id2 (Figure 2). From a screen of cyclin D1, cdk4, Rb and CDKIs, it was seen that ectopic expression of Id2 decreased p27KIP, as well as p15INK4b. The downregulation of p27KIP by overexpression of Id2 would account for the increased growth rate of cells ectopically expressing Id2 since p27KIP inhibits the cyclin D–cdk4 complex allowing for cells to arrest in G1. Ectopic Id2 expression induced a more robust decrease in p27KIP protein levels compared with the decrease in p27KIP RNA, indicating that transcriptional as well as posttranscriptional and posttranslational mechanisms may be involved. One such modification is phosphorylation by cdk2 at T187, which is known to control the activity of p27KIP as well as its localization within the cell (34). However, since Id2 had no effect on the levels of ectopically expressed p27KIP (Figure 5), Id2 may not control steady-state p27KIP protein levels in our system.

Similar to endogenous p27KIP RNA levels, promoter activity was significantly lower in cells ectopically expressing Id2. Chromatin immunoprecipitation and EMSA analysis revealed that Id2 reduced binding of E12/E47 (Figure 3D), shown previously to contribute to p27KIP induction (35), most probably by directly binding and sequestering E12/E47 via the HLH domain of Id2 (36,37). This would account for the failure of mutants lacking the HLH to suppress p27KIP (Figure 5), increase S phase and induce tumorigenesis (Figure 4). Previous studies showed that the Id2 HLH domain is also important for interaction with the pocket proteins Rb, p107 and p130 (26), as well as indirectly regulating other CDKIs, including p15 (38,39). While contributions from these pathways cannot be ruled out, our studies indicate a major role for p27. Interestingly, it was recently reported that Id3 also represses p27KIP, and this effect was mediated by the ETS transcription factor Elk1 (40) or E12/NeuroD2 (41). Induction of Id1 and Id3 mediated by latent membrane protein 1 of Epstein-Barr virus was also shown to downregulate p27KIP and cdk2 in rodent fibroblast transformation (27). We have also found previously that Id2 interacts with ETS factors Fli1 and Erg1/2 to regulate its own promoter (16).

**Discussion**

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To determine if changes in proliferation and focus formation (Figure 2) in vitro would reflect an in vivo model, cells were grafted or subcutaneously injected into nude mice. No tumors were formed by Id2−/− cells, whereas substantial tumors were evident on animals injected with cells ectopically expressing Id2 (Figure 2). From a screen of cyclin D1, cdk4, Rb and CDKIs, it was seen that ectopic expression of Id2 decreased p27KIP, as well as p15INK4b. The downregulation of p27KIP by overexpression of Id2 would account for the increased growth rate of cells ectopically expressing Id2 since p27KIP inhibits the cyclin D–cdk4 complex allowing for cells to arrest in G1. Ectopic Id2 expression induced a more robust decrease in p27KIP protein levels compared with the decrease in p27KIP RNA, indicating that transcriptional as well as posttranscriptional and posttranslational mechanisms may be involved. One such modification is phosphorylation by cdk2 at T187, which is known to control the activity of p27KIP as well as its localization within the cell (34). However, since Id2 had no effect on the levels of ectopically expressed p27KIP (Figure 5), Id2 may not control steady-state p27KIP protein levels in our system.

Similar to endogenous p27KIP RNA levels, promoter activity was significantly lower in cells ectopically expressing Id2. Chromatin immunoprecipitation and EMSA analysis revealed that Id2 reduced binding of E12/E47 (Figure 3D), shown previously to contribute to p27KIP induction (35), most probably by directly binding and sequestering E12/E47 via the HLH domain of Id2 (36,37). This would account for the failure of mutants lacking the HLH to suppress p27KIP (Figure 5), increase S phase and induce tumorigenesis (Figure 4). Previous studies showed that the Id2 HLH domain is also important for interaction with the pocket proteins Rb, p107 and p130 (26), as well as indirectly regulating other CDKIs, including p15 (38,39). While contributions from these pathways cannot be ruled out, our studies indicate a major role for p27. Interestingly, it was recently reported that Id3 also represses p27KIP, and this effect was mediated by the ETS transcription factor Elk1 (40) or E12/NeuroD2 (41). Induction of Id1 and Id3 mediated by latent membrane protein 1 of Epstein-Barr virus was also shown to downregulate p27KIP and cdk2 in rodent fibroblast transformation (27). We have also found previously that Id2 interacts with ETS factors Fli1 and Erg1/2 to regulate its own promoter (16).
Transient overexpression of p27KIP1 reversed the hyperproliferative phenotype induced by Id2, indicating that p27 downregulation plays a major role downstream of Id2. Mutants containing the HLH domain of Id2 (AN37 and ΔC58) failed to induce hyperproliferation and tumorigenesis and did not have any suppressive effect on the p27KIP1 promoter, highlighting the importance of the HLH domain in mediating the upregulation of Id2 and concomitant downregulation of p27KIP1, which is a potential mechanism for neoplastic transformation and tumorigenesis. Id2 may therefore represent a therapeutic target for the control of unregulated proliferation and transformation occurring in cancerous cells.

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References

7. CooperSmith, C.M. et al. (1997) Gamma-ray-induced apoptosis in transgenic mice with proliferative abnormalities in their intestinal epithelium: re-entry of villus enterocytes into the cell cycle does not affect their radioresistance but enhances the radiosensitivity of the crypt by inducing p53. Oncogene, 15, 131–141.