Chemical-genetic analysis of cyclin dependent kinase 2 function reveals an important role in cellular transformation by multiple oncogenic pathways

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Edited by William G. Kaelin, Jr., Harvard Medical School, Boston, MA, and approved March 8, 2012 (received for review July 14, 2011)

A family of conserved serine/threonine kinases known as cyclin-dependent kinases (CDKs) drives orderly cell cycle progression in mammalian cells. Prior studies have suggested that CDK2 regulates S-phase entry and progression, and frequently shows increased activity in a wide spectrum of human tumors. Genetic KO/knockdown approaches, however, have suggested that lack of CDK2 protein does not prevent cellular proliferation, both during somatic development in mice as well as in human cancer cell lines. Here, we use an alternative, chemical-genetic approach to achieve specific inhibition of CDK2 kinase activity in cells. We directly compare small-molecule inhibition of CDK2 kinase activity with siRNA knockdown and show that small-molecule inhibition results in marked defects in proliferation of nontransformed cells, whereas siRNA knockdown does not, highlighting the differences between these two approaches. In addition, CDK2 inhibition drastically diminishes anchorage-independent growth of human cancer cells and cells transformed with various oncogenes. Our results establish that CDK2 activity is necessary for normal mammalian cell cycle progression and suggest that it might be a useful therapeutic target for treating cancer.

analog-sensitive kinase | cyclin-dependent kinase

During eukaryotic cell division, cyclin-dependent kinase (CDK) activity initiates both the DNA synthesis (S) phase and mitosis. Based on the timing of its activation and its cyclin-binding preferences, CDK2 is suspected to be responsible for facilitating the G1-S transition and initiating DNA synthesis as well as controlling the exit from S phase (1). This has been supported by studies showing that a dominant-negative form of CDK2 attenuates growth of cells in culture (2) and microinjected antibodies against CDK2 block S-phase initiation in mammalian cells (3). Because of its role in cell cycle control, CDK2 has been extensively studied in the context of cancer. Indeed, CDK2 expression and activity have been implicated in a variety of cancers (4–7). Furthermore, overexpression of CDK2 binding partners, cyclins E and A, can participate in the transformation of cells (8, 9). It has also been shown that cyclin E-deficient cells are resistant to oncogenic transformation (10) and that its overexpression accentuates tumor formation in mice (11, 12). Additionally, elevated cyclin E expression in several tumor types correlates with a worse prognosis for patients (13, 14). Thus, there has been considerable interest in the development of small-molecule inhibitors of CDK2 as a potential therapy for various cancers (15).

Recent studies, however, have suggested that CDK2 may have a redundant role in regulating cell cycle progression, challenging whether CDK2 would be an effective therapeutic target. Genetic ablation of CDK2 has little effect on cellular proliferation during early murine development (16–18). Furthermore, depletion of CDK2 using siRNAs or antisense oligonucleotides has little effect on the proliferation rates of various colon cancer lines (19). Thus, it was concluded that CDK2 is dispensable for cellular proliferation. These approaches, however, result in ablation of CDK2 protein expression, potentially allowing for compensation by other CDKs (20), and are therefore likely to have different effects than acute inhibition of CDK2 kinase activity using small molecules. Prior attempts to inhibit CDK2 kinase activity in vivo have relied on pan-CDK small-molecule inhibitors that are not entirely selective for any single CDK (reviewed in 15). Therefore, it remains unknown whether specific and acute inhibition of CDK2 activity can attenuate cellular proliferation or cellular transformation in the context of oncogenic signaling. In this report, we use a chemical genetic approach in which we replace endogenous WT CDK2 ($CDK2^{WT}$) in transformed mouse embryonic fibroblasts (MEFs) and human colon cancer cells with an analog-sensitive (AS) version that is mutated to allow for acute and selective inhibition using modified ATP analogs. This approach has been used recently to identify a distinct, nonredundant role of CDK2 in cellular proliferation of nontransformed cells (21). Here, we show that small-molecule inhibition of CDK2 also disrupts cellular growth of transformed MEFs and human colon cancer cells, defining a role for CDK2 in cellular proliferation under the control of oncogenic signaling.

Results

AS CDK2 Forms Active Complexes in Vitro and Can Be Selectively Inhibited by Modified ATP Analogs. A chemical-genetic approach to modify kinases so they can be selectively inhibited by engineered ATP analogs (22) involves mutating a bulky amino acid residue within the ATP binding site of a kinase to a smaller residue, thus creating a unique expanded pocket (Fig. 1*A*). Small molecules can be designed to fit in the newly engineered pocket, allowing them to inhibit the modified kinase selectively without affecting other mammalian kinases that lack the AS mutation (22). Reconstitution of baculovirus-produced and -purified AS CDK2 ($CDK2^{AS}$) and cyclin A followed by an in vitro kinase activity assay revealed that $CDK2^{AS}$ can be selectively inhibited by a pyrazolopyrimidine derivative 1NM-PP1 (IC_{50} = 5 nM),

Author contributions: D.H., N.E.H., R.P.F., and A.G. designed research; D.H., N.E.H., L.K., L.W., K.A.M., K.J.C., and A.G. performed research; L.W., K.A.M., C.Z., K.M.S., R.P.F., and A.G. contributed new reagents/analytic tools; D.H., N.E.H., L.K., K.A.M., R.P.F., and A.G. analyzed data; and D.H., N.E.H., and A.G. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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See Author Summary on page 6372 (volume 109, number 17).

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1111317109/-/DCSupplemental.

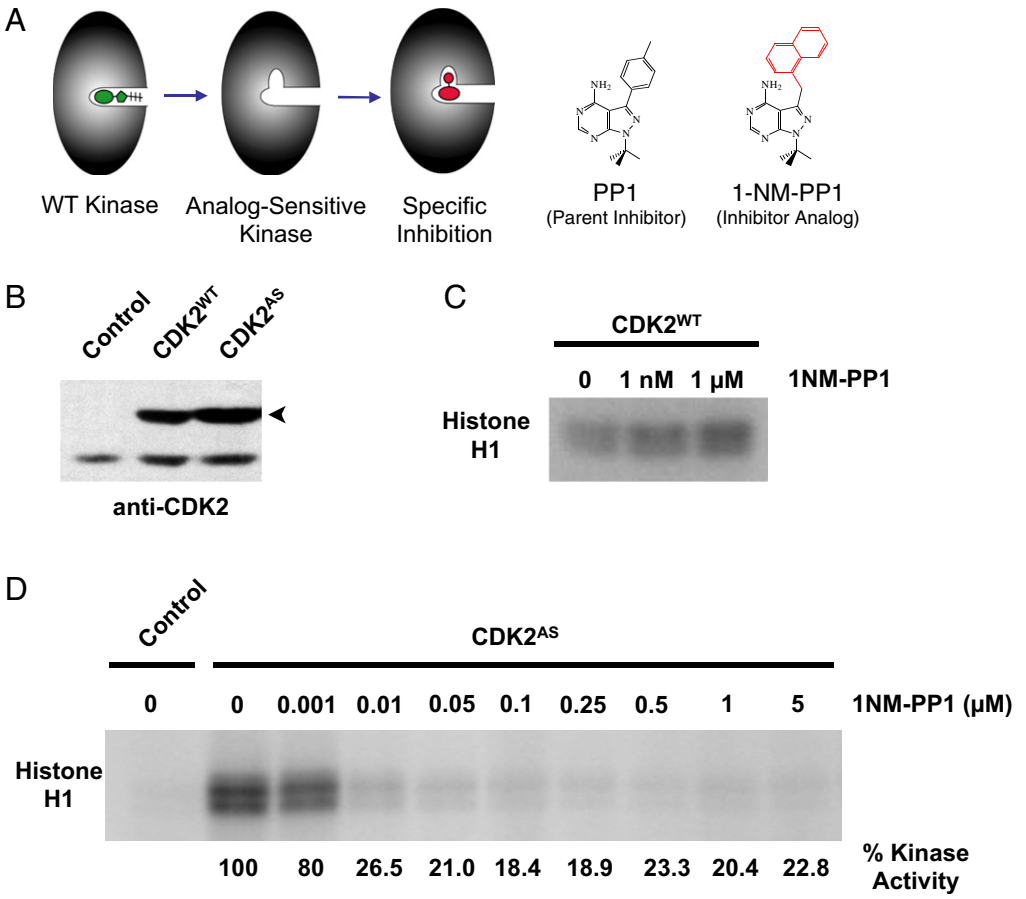

Fig. 1. CDK2^{AS} is catalytically active and can be potently inhibited by the selective inhibitor 1NM-PP1. (*A*) Model demonstrates the engineered CDK2^{AS} with the new binding pocket generated by the F80G mutation and the corresponding chemical inhibitor, 1NM-PP1, to inhibit the kinase selectively. (*B*) Expression of HA-tagged CDK2^{WT} or CDK2^{AS} protein in HEK 293T cells. Western blot shows expression of tagged (arrow) and endogenous CDK2 proteins. (*C*) CDK2^{WT} was immunoprecipitated from HEK 293T cell lysates with anti-HA antibodies, and kinase activity toward the histone H1 substrate was assessed in the presence of increasing amounts of 1NM-PP1. (*D*) CDK2^{AS} was immunoprecipitated from HEK 293T cell lysates with anti-HA antibodies, and kinase activity toward the histone H1 substrate was assessed in the presence of increasing amounts of 1NM-PP1.

whereas the IC_{50} of 1NM-PP1 for CDK2^{WT} is ~6,000-fold higher (29 μM) (22).

We asked if CDK2^{AS} could assemble an active complex with cyclins in vivo. The human CDK2^{AS} or CDK2^{WT} cDNA was tagged at the 3′ end with an HA epitope sequence and transfected into HEK cells (Fig. 1*B*). CDK2^{WT} or CDK2^{AS} protein was immunoprecipitated from whole-cell lysates with anti-HA antibodies, and kinase activity toward the histone H1 substrate was tested in the presence of increasing amounts of the 1NM-PP1 inhibitor (Fig. 1 *C* and *D*). Immunoprecipitated CDK2^{WT} was not appreciably inhibited by 1 μM 1NM-PP1, consistent with the purified protein studies (22) (Fig. 1*C*). In contrast, we found CDK2^{AS} to be inhibited at low nanomolar (~3–5 nM) 1NM-PP1 concentrations in the presence of 500 μM ATP (Fig. 1*D* and Fig. S1*A*). Thus, CDK2^{AS} is an active kinase when isolated from human cells and is extremely sensitive to a selective inhibitor.

Acute Inhibition of CDK2 Kinase Activity Results in Decreased Proliferation of MEFs, Whereas CDK2 Depletion with siRNA Does Not. To study the effects of acute CDK2 inhibition on cellular growth in vivo, we generated MEFs in which the endogenous mouse CDK2 is functionally replaced with human CDK2^{AS} or CDK2^{WT}. We used CDK2$^{Flox/Flox}$ MEFs [kindly provided by Marcos Malumbres and Mariano Barbacid (Central Nacional de Investigaciones, Madrid, Spain)] and deleted endogenous CDK2 by Cre-recombinase expression. Human CDK2^{AS} or CDK2^{WT} was then introduced by replication-defective retrovirus to generate two matched stable cell lines (Fig. S1 *B* and *C*). The MEFs continued to proliferate when cultured using the standard NIH 3T3 protocol, suggesting they underwent spontaneous immortalization. The p53 pathway appeared to remain intact, however, because treatment with the DNA damaging reagent doxorubicin induced expression of p21, a transcriptional target of p53 (Fig. S1*D*). In primary human tumors, CDK2 abundance is frequently found to be elevated severalfold, and its associated kinase activity may be elevated up to 40-fold compared with nontumorigenic tissues (4–7, 23). Both CDK2^{AS} and CDK2^{WT} MEFs expressed human protein ~10-fold higher than endogenous mouse CDK2 (24) (Fig. S1 *E* and *F*).

Using the AS allele of CDK2, we compared the effects of acute CDK2 kinase inhibition with siRNA knockdown of CDK2. MEFs expressing human CDK2^{WT} and CDK2^{AS} proliferate at comparable rates, with cell numbers increasing exponentially over a period of 4 d (Fig. 2*A*). On treatment of the cells with 1NM-PP1, CDK2^{AS} MEFs showed a dramatic decrease in proliferation, whereas the treatment of MEFs expressing CDK2^{WT} had no effect (Fig. 2*A*; $P < 0.00001$). Thus, 1NM-PP1 only affects the proliferation of cells that express the CDK2^{AS} allele, demonstrating the specificity of the chemical-genetic approach.

In contrast to treatment with 1NM-PP1, CDK2 knockdown with siRNAs resulted in an increase in proliferation of MEFs (Fig. 2*B*). We reasoned that this effect might be attributable to

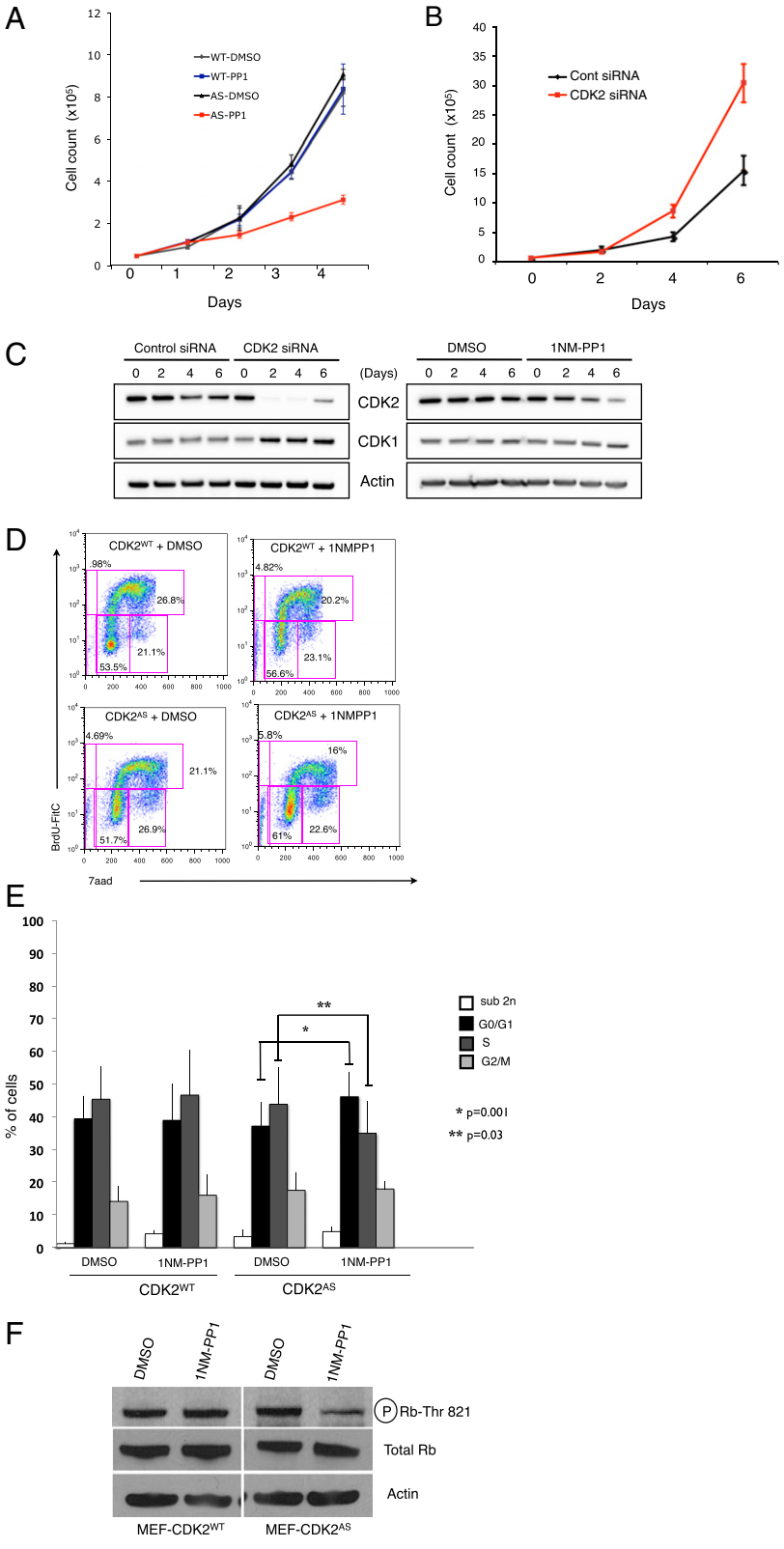

Fig. 2. Small-molecule inhibition, rather than siRNA-mediated knockdown, of CDK2 in MEFs decreases cellular proliferation. (*A*) Growth curve of MEFs carrying either the WT or AS allele of the CDK2 gene. The cells were treated at day 0 with 5 μM 1NM-PP1 or DMSO, and the cell number was counted at each time point. Growth curves represent the average of five independent experiments. The error bars represent mean ± SEM. Proliferation of the CKD2^{AS} cells in the presence or absence of 1NM-PP1 on both days 3 and 4 ($P < 0.00001$; two-tailed t test) is shown. (*B*) Growth curve of MEF-CDK2^{AS} cells treated with either non-targeting control siRNA or CDK2-specific siRNA. The cells were transfected with designated siRNAs, and the cell number was counted at each time point. Growth curves represent the average of three independent experiments. The error bars represent mean ± SEM. The statistical significance applies to both days 4 and 6 ($P < 0.00001$; two-tailed t test). (*C*) Western blot analysis of CDK expression in MEFs treated with either control/CDK2 siRNA or with DMSO/1NM-PP1. The cells were treated with a given reagent on day 0 and collected at each time point. (*D*) Cell cycle profiles determined for MEFs containing either the WT or AS allele of CDK2 by FACS analysis with pulse-BrdU incorporation for 45 min, after treatment with DMSO or 5 μM PP1 for 72 h. Cells were dual-stained with an FITC-conjugated anti-BrdU antibody (*y* axis) and 7-AAD to look at DNA content (*x* axis). Gated populations represent dead (sub2n), G0/G1, S, and G2/M cell populations. The depicted results are representative of four different experiments. (*E*) Quantification of cell cycle profiles. The error bars represent mean ± SEM. *Statistical significance as determined by a paired two-tailed *t* test. (*F*) Rb Thr821 phosphorylation following 36 h of 1NM-PP1 treatment of MEF CDK2^{WT} or MEF CDK2^{AS} cells. The cells were first serum-starved for 90 h in the presence of 0.1% serum and subsequently released into and grown for an additional 36 h in the medium containing 10% (vol/vol) serum and DMSO or 1NM-PP1.

functional compensation by another CDK, namely, CDK1, because CDK1 has been shown to be capable of replacing all the other interphase CDKs (20). To investigate this possibility, we examined protein levels of CDK1 and CDK2 following treatment with siRNAs against either CDK2 or 1NM-PP1. We saw a dramatic decrease in CDK2 protein levels on treatment with CDK2 siRNA, indicating that efficient knockdown had occurred (Fig. 2*C*). Interestingly, we also saw a greater than 75% increase in CDK1 protein levels within 48 h of CDK2 siRNA treatment, which paralleled the decrease in CDK2 protein expression. These results suggested that compensation by CDK1 might be responsible for the increased proliferation rates observed after

CDK2 knockdown. In contrast, treatment of CDK2^{AS} MEFs with 1NM-PP1 resulted in no appreciable decrease of CDK2 protein after 48 h (Fig. 2*C*). Prolonged inhibitor treatment (6 d), however, did demonstrate a modest decrease in CDK2 expression (Fig. 2*C*). Following 1NM-PP1 treatment, CDK1 levels remained relatively unchanged over the first 4 d of treatment but showed an approximate 33% increase, which paralleled the decrease in CDK2, after 6 d of treatment (Fig. 2*C*). We therefore conclude that acute inhibition of CDK2 disrupts MEF cell proliferation, whereas CDK2 knockdown does not, and this is likely attributable to a lack of compensation by CDK1 when CDK2 kinase activity is acutely inhibited.

We next asked whether the decrease in proliferation observed after acute CDK2 inhibition was attributable to decreased and/or delayed transition into S phase. CDK2^{AS} and CDK2^{WT} MEFs were treated with either the vehicle (DMSO) or the selective inhibitor (1NM-PP1), and BrdU incorporation was determined (Fig. 2 *D* and *E*). The CDK2^{AS} MEFs showed a significantly decreased percentage of cells in S phase and an increased percentage of cells in G1 when treated with 1NM-PP1(Fig. 2 *D* and *E*). Additionally, MEFs were synchronized by serum starvation and released into media containing 10% serum (vol/vol) with or without 1NM-PP1. We found that 24 h after the cells were released, 1NM-PP1 treatment resulted in ∼55% decreased phosphorylation of the retinoblastoma protein (Rb) (Fig. 2*F*). We therefore concluded that specific inhibition of CDK2 kinase activity leads to a delayed transition from G1 to S phase.

Acute Inhibition of CDK2 Results in Decreased Proliferation in both Anchorage-Dependent and Anchorage-Independent Growth of Human Colon Cancer Cells. We next asked whether acute inhibition of CDK2 could decrease proliferation of human cancer cells. HCT116 cells, a human colon cancer cell line, harbor a constitutively active mutant KRAS (G13N) (25) and also show a twofold increase in MYC expression compared with more differentiated colon cancer cells (26). Importantly, HCT116 cells are amenable to homologous recombination for genetic ablation or replacement of candidate genes (27). We used engineered HCT116 cells, in which the CDK2^{AS} alleles were inserted into both endogenous CDK2 loci (HCT116-CDK2^{AS}), replacing the endogenous WT gene by the method previously described for human CDK7 (21, 28). We first asked whether acute inhibition of CDK2 could decrease proliferation of the HCT116 cells. We found that in the absence of 1NM-PP1, HCT116-CDK2^{AS} cells exhibit a modest proliferation defect compared with WT HCT116 cells (Fig. 3*A*), which is consistent with what has been previously reported (21). On treatment with 1NM-PP1, we found that proliferation of HCT116-CDK2^{AS} cells was further decreased, whereas we saw no effect in the HCT116-CDK2^{WT} cells (Fig. 3*A*), suggesting that similar to the MEFs, acute inhibition of CDK2 significantly decreases proliferation of human cancer cells. In this system, the treatment of the AS cells with 1NM-PP1 did not result in the induction of cell death as assessed by PARP cleavage (Fig. S2). Additionally, we found that treatment of HCT116-CDK2^{AS} cells with 1NM-PP1 resulted in an increase of cells in G1 and a decrease in cells in S phase when subjected to a BrdU incorporation assay (Fig. 3 *B* and *C*). To ensure that the proliferation defect observed in HCT116-CDK2^{AS} cells in the absence of 1NM-PP1 did not render the AS allele more sensitive to general CDK2 inhibition, we treated HCT116-CDK2^{AS} and HCT116-CDK2^{WT} cells with a commercially available small-molecule CDK2 inhibitor, CVT-313 (29). We found that CVT-313 treatment resulted in a significant reduction in cells in S phase in both HCT116-CDK2^{AS} and HCT116-CDK2^{WT} cell lines, to an equal extent (Fig. S3), indicating that the AS mutation did not increase sensitivity to other CDK2 inhibitors.

We next determined whether CDK2 kinase activity is essential for anchorage-independent growth, a hallmark of cellular transformation. Both HCT116-CDK2^{WT} and HCT116-CDK2^{AS} cells formed visible colonies within 3 wk of growth in soft agar (Fig. 3*D*). In contrast, when the AS line was treated with 1NM-PP1, the number and size of colonies that formed were drastically reduced (Fig. 3 *D* and *E*), thus indicating that CDK2 is necessary for anchorage-independent growth of a human cancer cell line.

As a second approach to examine anchorage-independent growth, we used a tumorsphere formation assay, which examines colony growth under nonadherent culture conditions (30). An advantage of this approach is that cells can be recovered from tumorspheres for cell cycle analysis and protein extraction. HCT116 cells formed visible tumorspheres within 10 d when grown on ultra-low-attachment plates, as has been previously reported (31). Similar to the soft agar assay, we found that 1NM-PP1 treatment reduced the size and number of tumorspheres formed from HCT116-CDK2^{AS} cells (Fig. 3*F*). This result could be quantified using a luminescence-based assay to measure the amount of ATP in an individual well, which corresponds to the number of live cells (Fig. 3*G*). We next asked whether 1NM-PP1 treatment affected cell cycle distribution of the proliferating tumorspheres. When exposed to BrdU for 24 h, we found that HCT116-CDK2^{AS} tumorspheres grown in the presence of 1NM-PP1 showed a significant decrease of cells in S phase (Fig. 3 *H* and *I*). These results confirm that acute CDK2 kinase inhibition attenuates anchorage-independent growth of an established human cancer cell line.

CDK2 Is Required for Anchorage-Independent Growth but Not Monolayer Growth in MEFs Transformed with a Variety of Oncogenes. HCT116 tumor cells harbor multiple oncogenic events, including a KRAS activating mutation and MYC overexpression (25, 26). To study the consequence of CDK2 inhibition in a more defined genetic background, we used MEFs, which are amenable to cellular transformation by diverse oncogenic signals. We infected CDK2^{WT} and CDK2^{AS} MEFs with replication-defective retrovirus to express human MYC, activated HRAS, or v-ABL (Fig. 4*A*), three well-characterized oncogenes. As with the untransformed MEFs, we observed that the p53 pathway remained intact in these transformed cells, as evidenced by p21 induction following treatment with the DNA damaging agent doxorubicin (Fig. S4*A*).

We first sought to determine if overexpression of each of the three oncogenes could alter cellular proliferation in the context of acute CDK2 inhibition. Interestingly, in contrast to non-transformed CDK2^{AS} MEFs and HCT116-CDK2^{AS} cells, treatment with 1NM-PP1 did not appreciably alter cell proliferation of the transformed MEFS, regardless of which oncogene was overexpressed (Fig. 4*B*). These observations suggest that overexpression of individual oncogenes can decrease sensitivity of MEFs to CDK2 inhibition.

In contrast to the monolayer growth of cultured cells, 3D anchorage-independent growth of transformed cells in soft agar is known to be critically dependent on the abundance of cyclin E and A expression (8, 32, 33), the activating subunits of CDK2. We therefore asked whether growing the transformed MEFs in nonadherent culture conditions would increase their sensitivity to CDK2 inhibition. When grown in soft agar, all three of the oncogene-transformed cell lines acidified the media and formed colonies within 3 wk (Fig. S4*B*). In contrast, when AS cell lines were treated with 1NM-PP1, the number and size of colonies that formed were drastically reduced regardless of which oncogene was used to transform the cells (Fig. 4*C* and Fig. S4*B*). Furthermore, when transformed MEFs were grown on ultra-low-attachment plates, 1NM-PP1 significantly reduced the amount of tumorspheres that formed (Fig. 5*A*). These results indicate that although it has little effect on cells grown in a monolayer, acute specific inhibition of CDK2 can significantly reduce anchorage-

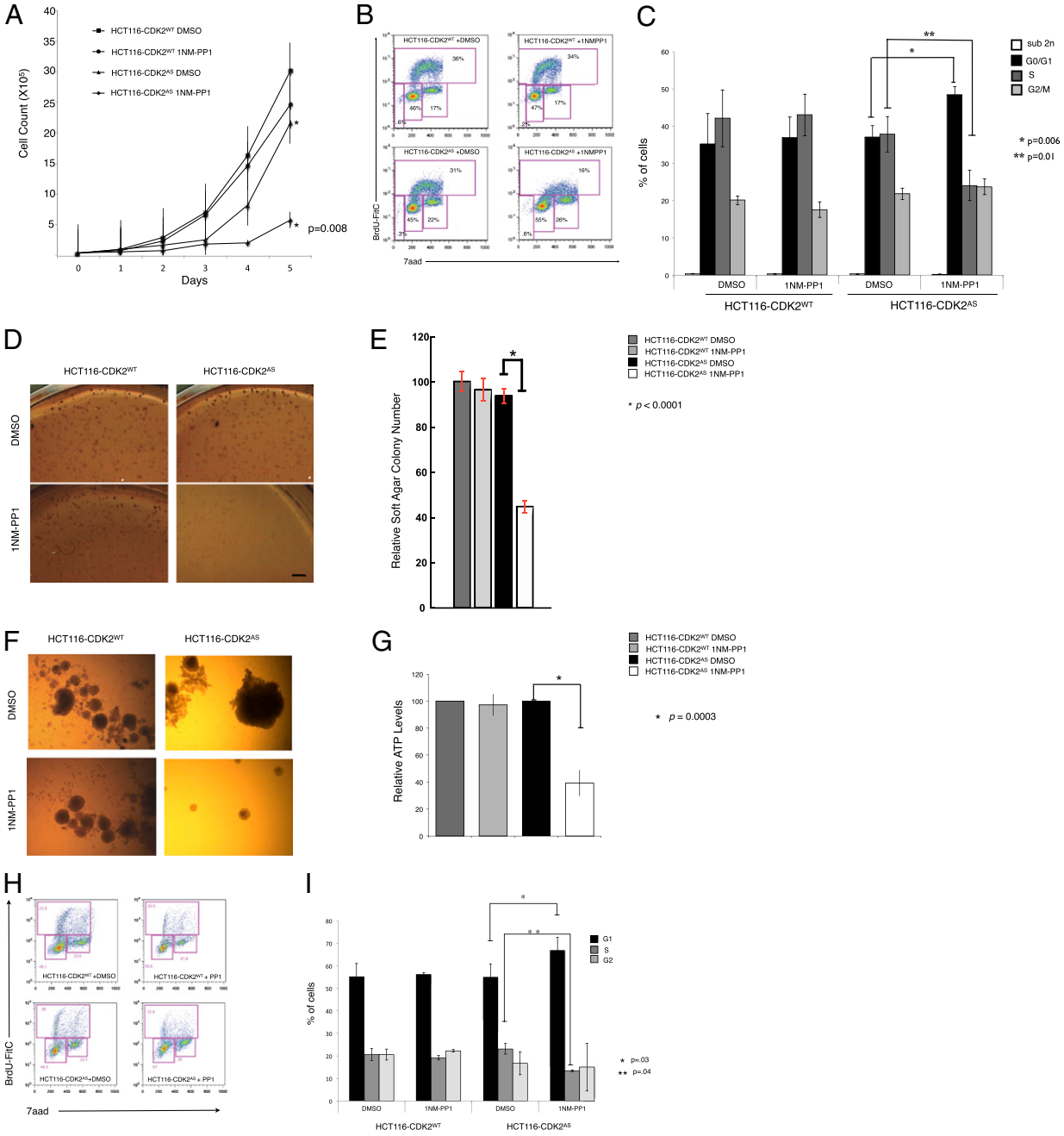

Fig. 3. Diminished anchorage-independent growth and proliferation of HCT116 colon cancer cells following acute CDK2 inhibition. (*A*) Growth curve of HCT116 cells with either a WT or AS allele of CDK2 gene. The cells were treated on day 0 with 5 μM 1NM-PP1 or DMSO, and the cell number was counted at each time point. Growth curves represent the average of three independent experiments. The error bars represent mean ± SEM. Proliferation of the CKD2^{AS} cells in the presence or absence of 1NM-PP1 ($P = 0.008$; two-tailed t test) is shown. (*B*) Cell cycle profiles for HCT116 cells containing either the WT or AS allele of CDK2, determined by FACS analysis with pulse-BrdU incorporation for 45 min after treatment with DMSO or 5 μM PP1 for 72 h. Cells were dual-stained with an FITC-conjugated anti-BrdU antibody (*y* axis) and 7-AAD to examine DNA content (*x* axis). Gated populations represent dead (sub2n) and G0/G1, S, and G2/M cell populations. The depicted results are representative of four different experiments. (*C*) Quantification of cell cycle profiles. The experiment was repeated four times. *Statistical significance as determined by a paired two-tailed t test. The error bars represent mean ± SEM. (*D*) Anchorage-independent growth of HCT116 cells in soft agar. HCT116 cells were pretreated with DMSO or 5 μM 1NM-PP1 for 72 h. Following pretreatment, cells were plated in 0.32% soft agar containing DMSO or 5 μM 1NM-PP1. (Scale bar = 5 mm.) (*E*) Quantification of colony growth in soft agar after 21 d. The error bars represent mean ± SEM. (*F*) Tumorsphere formation of HCT116 cells. HCT116 cells were seeded into ultra-low-attachment plates in media containing DMSO or 5 μM 1NM-PP1. (Magnification: 40×.) (*G*) Quantification of tumorsphere formation after 14 d. The amount of live cells per well was quantified using a luminescence-based assay to determine levels of ATP. 1NM-PP1–treated wells were normalized to DMSO-treated wells for each cell line independently. The experiment was repeated five times. *Statistical significance as determined by a two-tailed t test. The error bars represent mean ± SEM. (*H*) Cell cycle profiles for tumorspheres formed from HCT116 cells after 10 d of growth in DMSO or 5 μM 1NM-PP1, determined by FACS analysis with pulse-BrdU incorporation for 24 h. Cells were dual-stained with an FITC-conjugated anti-BrdU antibody (*y* axis) and 7-AAD to examine DNA content (*x* axis). Gated populations represent G0/G1, S, and G2/M cell populations. The depicted results are representative of three different experiments. (*I*) Quantification of cell cycle profiles. The experiment was repeated three times. *Statistical significance as determined by a paired two-tailed t test. The error bars represent mean ± SEM.

independent growth, a hallmark of cancer cells, regardless of which of these oncogenes is driving cellular transformation.

Altered Expression of Cell Cycle Regulators in MEFs Cultured in Nonadherent Conditions Is Associated with Increased Sensitivity to CDK2 Kinase Inhibition. We next asked why the transformed MEFs were more sensitive to CDK2 inhibition when grown in soft agar and nonadherent culture conditions. It has been shown that cells exhibit significant transcriptional alterations, including the expression of cell cycle proteins, when grown in adherent vs. nonadherent culture conditions (8, 32, 34–37). Thus, we hypothesized that the observed sensitivity of the oncogene-transformed MEFs to a selective small-molecule inhibitor of CDK2 could be attributable to altered expression of cell cycle proteins. We examined the expression levels of cell cycle proteins in cells grown in adherent vs. nonadherent culture conditions. We found that in each of the transformed lines, protein expression of all the cyclins, with the exception of cyclin E in the MYC-transformed MEFs, was significantly diminished in the nonadherent cells (Fig. 5*B*). Likewise, CDK1 and CDK2 expression was also diminished in the nonadherent cells (Fig. 5*B*). Interestingly, we also found that the endogenous inhibitors of cell cycle progression, p27 and p21, as well as the Rb tumor suppressor protein were markedly up-regulated in the nonadherent cells (Fig. 5*C*), suggesting that cell cycle control mechanisms are heightened in this context. Corresponding with a decrease in cyclin D1 and E1 expression, we also found a decrease in the inactivating phosphorylation of Rb (Fig. 5*C*). Similar results were observed when comparing HCT116 cells grown in adherent vs. nonadherent conditions (Fig. S5). Thus, both transformed MEFs and human tumor cells attenuate expression of proproliferative cell cycle proteins when grown in nonadherent conditions.

We next asked whether it was an individual cyclin whose down-regulation might render the transformed cells sensitive to CDK2 kinase inhibition in nonadherent conditions. To address this question, we used an RNAi approach to knock down individual cyclins in the transformed MEFs grown in adherent conditions and assessed their sensitivity to acute CDK2 inhibition. The cells were first treated with siRNA alone for 48 h to achieve cyclin knockdown (Fig. 5*D*). Cells were subsequently treated with 1NM-PP1 for 48 h (Fig. 5*D*). The influence of knocking down individual cyclins on the proliferation of transformed cells was examined 96 h after siRNA treatment. Knockdown of either cyclin D1 or B1 alone inhibited cell proliferation significantly (Fig. 5*E, Upper*). A role for cyclin D in oncogenic transformation has been previously established (38, 39). The pronounced effects of cyclin B1 knockdown are likely attributable to diminished CDK1 activity. Our observation may be attributable, at least in part, to the extent and persistence of cyclin knockdown that could be achieved with these siRNAs throughout the entire course of the experiment (Fig. 5*D*). Next, the oncogene-transformed $CDK2^{AS}$ MEFs were pretreated with siRNAs against individual cyclins, followed by treatment with 1NM-PP1. Combined siRNA and 1NM-PP1 treatment of transformed cells led to an additional significant reduction in proliferation. These results demonstrate that attenuating cyclin expression can sensitize transformed cells to acute selective CDK2 inhibition. (Fig. 5*E*, *Lower*). Thus, our results are consistent with the hypothesis that the increased sensitivity of transformed cells grown in soft-agar and tumorsphere conditions to CDK2 inhibition is attributable to an overall reduction in proproliferative cell cycle proteins (i.e., cyclins, CDKs) and the up-regulation of CDK inhibitors.

Discussion

We have generated an AS human CDK2 allele that can be potently, selectively, and acutely inhibited by a small-molecule inhibitor, 1NM-PP1. Using this allele, we have shown that acute small-molecule inhibition of CDK2 kinase activity but not CDK2 depletion dramatically attenuates growth of multiple cell types. These include nonmalignant MEFs and a human colon cancer cell line in which both endogenous CDK2 alleles are replaced with the $CDK2^{AS}$. Furthermore, abundant overexpression of multiple oncogenes appears to overcome the effects of CDK2 inhibition on cell proliferation in monolayer culture, but anchorage-independent growth in soft-agar or tumorsphere assays is still dramatically diminished when the oncogenes are overexpressed. These findings contrast with prior studies, which found that CDK2 was not required for the proliferation of several cancer cell lines (19). Our results emphasize that a fundamental difference exists between genetic loss or gene knockdown approaches and small-molecule inhibition of CDK2 kinase activity. Furthermore, our findings suggest that CDK2 inhibition may indeed have utility in the treatment of cancers driven by a wide variety of oncogenic signals.

Genetic ablation of *CDK2* using engineered KO mice shows that the mice are viable and embryonic fibroblasts derived from these mice exhibit relatively normal proliferation (17, 18). The modest effects observed in the CDK2 KO mice are likely attributable to compensation by other CDKs, such as CDK4/6 at the G1/S transition or CDK1 within the G1/S and G2 phases (20). For example, CDK1/cyclin E complexes are not detected until CDK2 expression is lost, indicating a switch whereby cyclin E preferentially binds to CDK2; however, in the absence of CDK2, cyclin E can be associated instead with CDK1 (20). In contrast to genetic loss, specific inhibition of CDK2 kinase activity by small-molecule inhibitors does not immediately allow the cell to compensate for the missing protein. The presence of an inhibitor-bound and inactive CDK2 protein prevents cyclin "switching" to other CDKs, such as CDK1.

We find that acute and selective inhibition of CDK2 can attenuate anchorage-independent growth of cells transformed by a variety of different oncogenes as well as human HCT116 tumor cells. Several prior studies found that expression of cyclins E and A, the activating subunits of CDK2, is rapidly down-regulated in cells grown in an anchorage-independent manner (8, 32, 34). In contrast, cyclin A overexpression in Rat1a cells is sufficient to induce anchorage-independent growth (8). Thus, CDK2 activity may be limiting in transformed cells grown in an anchorage-independent manner. Our results support this hypothesis, because the ability of three potent oncogenes to elicit anchorage-independent growth is substantially diminished following selective CDK2 inhibition (Fig. 4*C*).

Transformed cells lose contact inhibition, can adhere to one another, and form 3D colonies when grown in soft agar. Several prior studies have found that many conventional chemotherapeutics are less potent against tumor cells when they are grown in 3D cultures (40, 41). In contrast, we find that selective CDK2 inhibition preferentially blocks the proliferation of tumor cells in soft agar. Inhibition of CDK2 may therefore define a unique type of therapy that preferentially affects the anchorage-independent growth of tumor cells in 3D culture.

Prior attempts to inhibit CDK2 acutely in vivo have relied on small molecules that are not entirely selective for one CDK. Our chemical-genetic approach allows for truly selective inhibition of a kinase of interest with unparalleled specificity. We now show that acute inhibition of CDK2 alone is sufficient to diminish proliferation of normal and malignant cells. Our study challenges the notion that CDK2 is dispensable, and identifies it instead as a potentially useful therapeutic target for arresting the anchorage-independent proliferation of tumor cells driven by a variety of oncogenic signals.

Materials and Methods

In Vitro Kinase Assays. $CDK2^{WT}$ and $CDK2^{AS}$ cDNAs were tagged at the 3′ end with a HA epitope and transfected into HEK cells. Equal amounts of $CDK2^{WT}$ or $CDK2^{AS}$ proteins were immunoprecipitated from whole-cell lysates with

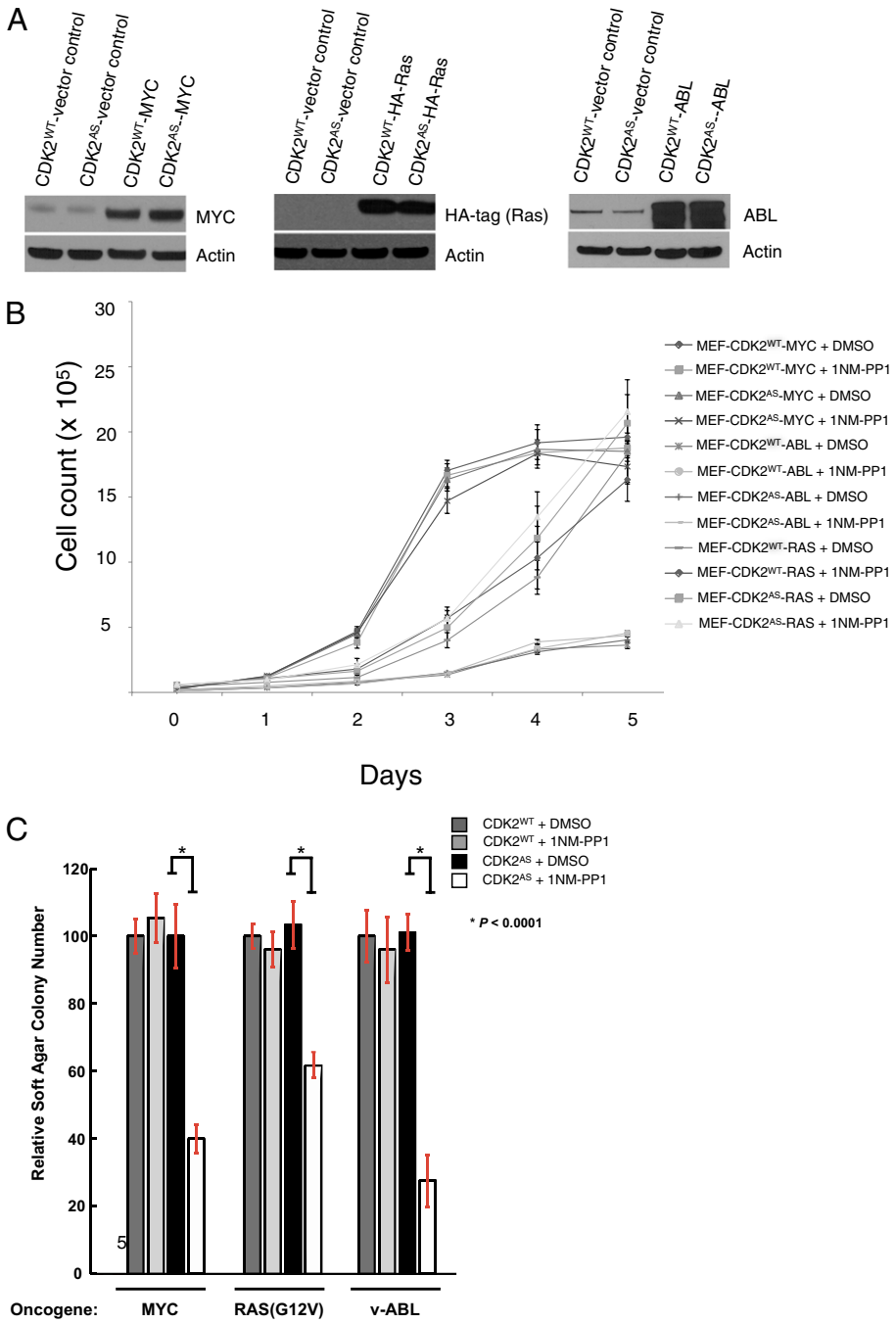

Fig. 4. CDK2 inhibition attenuates nonadherent colony formation but does not alter the growth of transformed MEFs in adherent conditions. (*A*) Western blot showing retroviral overexpression of the oncogenes MYC, RAS, and ABL in MEF-CDK2^{WT} or MEF-CDK2^{AS} cells. (*B*) Growth curve of the oncogene-transformed MEFs carrying either a WT or AS allele of CDK2 gene. The cells were treated at day 0 with 5 μM 1NM-PP1 or DMSO, and the cell number was counted at each time point. Growth curves represent the average of four independent experiments. The error bars represent mean ± SEM. Proliferation of the CKD2^{AS} cells in the presence or absence of 1NM-PP1 on days 3, 4, and 5 ($P > 0.05$) is shown. (*C*) Quantification of anchorage-independent growth in soft agar. The oncogene-transformed MEFs were grown in soft agar for 21 d in the presence of DMSO or 5 μM 1NM-PP1 and assessed for their sensitivity to CDK2 inhibition. The experiments were repeated at least three times in triplicate. The error bars represent mean ± SEM ($P < 0.0001$, two-tailed *t* test).

anti-HA antibodies, and kinase activity toward the histone H1 substrate was assessed in the presence of increasing amounts of the 1NM-PP1 inhibitor as previously described (22, 42).

Generation of CDK2^{WT} and CDK2^{AS} Cell Lines. MEFs in which the CDK2 allele is flanked by Lox-P recombination sites were provided by Mariano Barbacid's laboratory. Endogenous CDK2 was deleted using recombinant retrovirus expressing Cre-recombinase. Human CDK2^{WT} or CDK2^{AS} alleles were introduced into Cdk2$^{-/-}$ MEFs using retroviral transduction and selected for puromycin resistance. These cells were subsequently transformed by oncogenes c-MYC, HRASG12V, and v-ABL via retroviral transduction. HCT-116 CDK2^{WT} and CDK2^{AS} were generated by introducing human CDK2^{AS} alleles into the CDK2 locus using a recombinant adeno-associated virus (AAV) homologous recombination strategy that is described in detail elsewhere (21).

Cell Cycle Analysis. For BrdU incorporation experiments in adherent growth conditions, cells were treated with 5 μM 1NM-PP1 or diluent (DMSO) for 72 h and then incubated with 10 μM BrdU for 45 min. Cells were harvested using

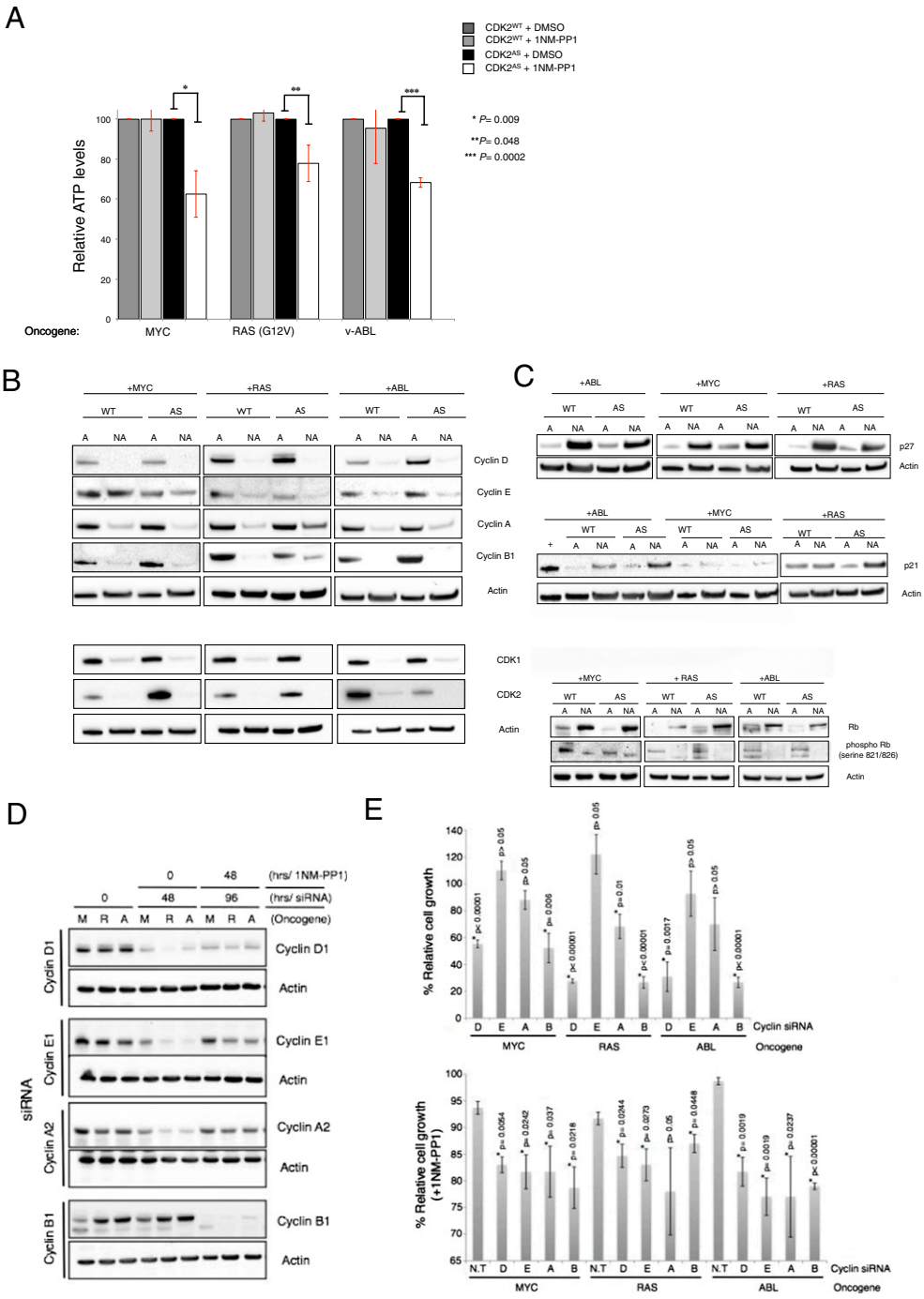

Fig. 5. Nonadherent growth of oncogene-transformed MEFs results in the altered expression of cell cycle proteins and is associated with increased sensitivity to CDK2 inhibition. (*A*) 1NM-PP1 treatment of the oncogene-transformed MEFs alters nonadherent growth. The oncogene-transformed MEFs were subjected to the tumorsphere assay, and cell growth was assessed by quantitation of ATP abundance after 10–14 d of growth. 1NM-PP1 wells were normalized to DMSO-treated wells for each individual cell line. *Statistical significance as determined by a two-tailed *t* test. The error bars represent mean ± SEM. The experiment was repeated three times with Abl-overexpressing cells and five times each with MYC- and RAS-overexpressing cells. (*B*) Western blot analysis of cyclins and CDK1 and CDK2 expression in the oncogene-transformed MEFs grown in either adherent (A) or nonadherent (NA) conditions. (*C*) Western blot analysis of tumor suppressors p27, p21, and Rb in the oncogene-transformed MEFs grown in either adherent or nonadherent conditions. (*D*) Western blot analysis of cyclin expression following siRNA-mediated knockdown of individual cyclins in oncogene-transformed MEFs. The transformed cells (M, MYC; R, RAS; A, ABL) were first treated with the siRNAs against indicated cyclins alone for 48 h and then further cultured for an additional 48 h in the presence or absence of 1NM-PP1. (*E*) (*Upper*) Effect of cyclin knockdown on the growth of the oncogene-transformed MEFs. The cells were treated with the siRNAs against the indicated cyclins alone for 96 h, and cell counts were performed. The extent of growth inhibition caused by knockdown of each individual cyclin was normalized to the nontargeted control siRNA-treated cells. (*Lower*) Effects of individual cyclin knockdown on the sensitivity of the oncogene-transformed cells treated with 1NM-PP1. The transformed cells were first treated with siRNAs against individual cyclins or nontargeting (N.T) control siRNA for 48 h and then further cultured for additional 48 h in the presence of DMSO or 1NM-PP1 before cell counting was performed. The proliferation of 1NM-PP1–treated cells was normalized to that of DMSO-treated control cells. These experiments were independently repeated three times in triplicate. The error bars represent mean ± SEM. *Statistical significance as determined by a two-tailed *t* test.

0.05% trypsin; they were then fixed, DNase-treated, and dual-stained with an FITC-conjugated BrdU antibody and 7-amino-actinomycin D (7-AAD) (to determine DNA content) using the BrdU Flow Kit from BD Pharmingen. Tumorspheres grown on ultra-low-adherent plates were exposed to 10 μM BrdU for 24 h to account for a slower proliferation rate and were dissociated to a single-cell suspension using 0.05% trypsin followed by mechanical dissociation using a 24-gauge syringe. Cells were fixed and stained using the same protocol for adherent cells. Cells were analyzed using an LSRII flow cytometer (BD Biosciences), and percentages in G1, S, and G2/M phases were determined using FlowJo (Tree Star, Inc.) analysis software. Cell death was accounted for by using 7-AAD staining to identify cells with DNA content less than 2 N. The two-tailed paired Student *t* test was used to determine the differences between groups.

Cell Proliferation Assays. A total of 5×10^4 MEFs were plated onto six-well plates in triplicate, and the time-course experiment was repeated five times. The cells were harvested at each time point, and the cell number was counted using Guava ViaCount reagent (Millipore) according to the manufacturer's instruction.

Anchorage-Independent Cellular Proliferation Assays. Anchorage-independent proliferation was determined by soft-agar colony growth and tumorsphere formation assays. For soft-agar growth, MEFs and HCT116 cells were treated with 5 μM 1NM-PP1 or DMSO for 72 h. After 72 h of pretreatment, cells were seeded at a density of 5×10^5 cells per well in a standard 6-well culture dish in 0.32% agar containing 5 μM 1NM-PP1 or DMSO. Cells were cultured for 21 d; at that point, colonies were counted. The two-tailed Student *t* test was used to determine significant differences between groups. For tumorsphere formation assays, MEFs and HCT116 cells were seeded at a density of 3×10^3 or 1.2×10^4 cells per well of 24-well or 6-well ultra-low-attachment plates (Corning), respectively. Cells were cultured between 10 and 14 d; at that point, cell quantification was performed for each well using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instruction.

siRNA Experiments. siRNAs against human CDK2; mouse cyclins D1, E1, A2, and B1, respectively; and a pool of nontargeting control siRNA (siGENOME SMART pool siRNA) were purchased from Dharmacon and used according to the manufacturer's protocol.

Protein Lysates and Western Blotting Analysis. Cultured cells were washed with ice-cold PBS and harvested directly into radioimmunoprecipitation assay buffer [50 mM Tris, 150 mM NaCl, 0.5% sodium-deoxycholate, 1% Nonidet P-40, 0.1% SDS, 2 mM EDTA (pH 7.5)] containing COMPLETE protease inhibitor mixture (Roche) and phosphatase inhibitors (Santa Cruz Biotechnology). Protein concentrations were determined by performing a Detergent-Compatible Protein Assay (Bio-Rad) using BSA as a standard. Quantification of Western blots was done using ImageJ (National Institutes of Health) densitometry analysis or a Bio-Rad ChemiDoc XRS+ Molecular Imager equipped with Image Lab software. The following antibodies were used for Western blot analyses: MYC (Epitomics), β-Actin (Sigma), PARP (Cell Signaling), Rb (clone 4.1.; University of Iowa Hybridoma Bank), Phospho-Thr821 Rb (Invitrogen), Phospho-Thr821/826 Rb (E-10; Santa Cruz Biotechnology), Phospho-Ser-807/811 Rb (Cell Signaling), CDK2 (D-12; Santa Cruz Biotechnology), CDK1 (Santa Cruz Biotechnology), cyclin D1 (DCS6; Cell Signaling), cyclin E (Millipore), cyclin A (C-19; Santa Cruz Biotechnology), cyclin B1 (GNS1; Thermo Scientific), p21 (BD Pharmingen), p27 (BD Transduction Laboratory), p19ARF (Clone 5-C3-1; Millipore), p53 (CM5; Leica Microsystems), HA-tag (6E2; Cell Signaling), and ABL (BD Pharmingen).

ACKNOWLEDGMENTS. We thank Drs. David Morgan and J. Michael Bishop for their kind support and advice during the early preparatory phases of this work. The work was supported by fellowship support (Grant WO1456) of the Deutsche Forschungsgemeinschaft (to L.W.) and the California Breast Cancer Research Program (D.H., N.E.H., and L.K.), and by National Institutes of Health Grants GM056985 (to R.P.F.), EB001987 (to K.M.S.), and CA136717 (to A.G.). We also acknowledge the support of the University of California, San Francisco Program for Breakthrough Biological Research and a V-Foundation Scholar award (to A.G.).

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