

# Mammalian Alteration/Deficiency in Activation 3 (*Ada3*) Is Essential for Embryonic Development and Cell Cycle Progression<sup>\*[5]</sup>

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**Background:** *Ada3* is a core component of HAT containing coactivator complexes.

**Results:** Germline deletion of *Ada3* is embryonic lethal, and cell deletion leads to abnormal cell cycle progression.

**Conclusion:** *Ada3* is a critical protein at organismic and cellular level.

**Significance:** This study describes a novel role of *Ada3*, a component of HAT complexes, as a critical regulator of cell survival.

*Ada3* protein is an essential component of histone acetyltransferase containing coactivator complexes conserved from yeast to human. We show here that germline deletion of *Ada3* in mouse is embryonic lethal, and adenovirus-Cre mediated conditional deletion of *Ada3* in *Ada3*<sup>FL/FL</sup> mouse embryonic fibroblasts leads to a severe proliferation defect which was rescued by ectopic expression of human *Ada3*. A delay in G<sub>1</sub> to S phase of cell cycle was also seen that was due to accumulation of Cdk inhibitor p27 which was an indirect effect of *c-myc* gene transcription control by *Ada3*. We further showed that this defect could be partially reverted by knocking down p27. Additionally, drastic changes in global histone acetylation and changes in global gene expression were observed in microarray analyses upon loss of *Ada3*. Lastly, formation of abnormal nuclei, mitotic defects and delay in G<sub>2</sub>/M to G<sub>1</sub> transition was seen in *Ada3* deleted cells. Taken together, we provide evidence for a critical role of *Ada3* in embryogenesis and cell cycle progression as an essential component of HAT complex.

The eukaryotic cell cycle progression depends on proper coordination of DNA replication and duplication of chromo-

somes to daughter cells (1), a process precisely regulated by modification of chromatin that allows the accessibility to factors involved in transcription (2). Thus, proteins involved in modulating the structure of chromatin play an important role in cell cycle progression. The post-translational modification of core histones (H2A, H2B, H3, and H4) is an essential process for altering chromatin structure (3, 4). Histone acetyltransferases (HATs)<sup>6</sup> and histone deacetylases are required to maintain steady state levels of acetylation (5). Several HAT enzymes, such as general control nonderepressible 5 (Gcn5), p300/CBP-associated factor (PCAF), p300, and CREB-binding protein (CBP), have been identified over the years (6, 7). Most of the HATs are part of large complexes such as the human TBP-free TAF complex (TFTC); the Spt3/Taf9/Gcn5 acetyltransferase complex (STAGA) (human homolog of yeast SAGA complex) and the *Ada2a*-containing (ATAC) complex that play a role in several important processes, such as cell cycle (8, 9). Additionally, previous studies from our laboratory and that of others have demonstrated the presence of p300 HAT in *Ada3*-containing protein complexes (10, 11). Given the combined presence of *Ada3* with Gcn5 in a number of distinct HAT complexes, recent evidence for a role of Gcn5 in regulating DNA replication as well as mitosis (12–14) suggest that *Ada3* may also play a role in cell cycle. Despite the range of established and potential cellular functions of *Ada3* as part of multiple HAT complexes, the *in vivo* physiological role of mammalian *Ada3* is not known.

We previously identified human *Ada3* as a novel human papillomavirus 16 E6-binding protein (15). Human *Ada3* is the

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[5] This article contains supplemental Materials and Methods, Figs. S1–S6, and Tables S1–S3.

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<sup>6</sup> The abbreviations used are: HAT, histone acetyltransferase; *Ada3*, alteration/deficiency in activation 3; *hAda3*, human *Ada3*; MEF, mouse embryonic fibroblast; Cdk, cyclin-dependent kinase; Gcn5, general control nonderepressible 5; PCAF, p300/CBP-associated factor; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; STAGA, Spt3/Taf9/Gcn5 acetyltransferase complex; ATAC, *Ada2a*-containing complex; adeno-Cre, adenovirus expressing the Cre recombinase; Rb, retinoblastoma protein; E, embryonic days; PI, propidium iodide; TBP, TATA-binding protein; TAF, TBP-associated factor.

homologue of the yeast Ada3, an essential component of the Ada transcriptional coactivator complex composed of Ada2, Ada3, and a HAT component Gcn5 (16). Genetic studies in yeast have demonstrated that Ada3 functions as a critical component of coactivator complexes that link transcriptional activators, bound to specific promoters, to histone acetylation and basal transcriptional machinery (17–19). We showed that Ada3 binds and stabilizes the tumor suppressor p53 protein and is required for p53 acetylation by p300 (20). Work from our laboratory has also shown that Ada3 is required for HAT recruitment to estrogen receptors and their transcription activation function (11). We and others have shown that Ada3 also associates with and regulates transcriptional activity of other nuclear hormone receptors, including retinoic acid receptor (21) and androgen receptor (22).

Here, we used conditional deletion of mouse *Ada3* gene to explore the physiological importance of mammalian Ada3. We demonstrate that homozygous deletion of *Ada3* is early embryonic lethal. *Ada3* deletion in *Ada3<sup>Flox/Flox</sup>* (*Ada3<sup>FL/FL</sup>*) MEFs showed that Ada3 is required for efficient cell cycle progression through G<sub>1</sub> to S transition as well as for proper mitosis. Detailed analyses in this system revealed an Ada3-c-Myc-Skp2-p27 axis that controls G<sub>1</sub> to S phase progression and partly contributes to cell cycle delay upon *Ada3* deletion. Additionally, loss of *Ada3* showed dramatic decrease in acetylation of core histones that are known to play an important role in cell cycle. Loss of *Ada3* also resulted in several changes in gene expression as observed by microarray analyses. Notably, many of the genes affected were involved in mitosis. Taken together, we present evidence for an essential role of mammalian *Ada3* in embryonic development and cell cycle progression.

## EXPERIMENTAL PROCEDURES

**Generation of *Ada3* Gene-targeted Mice, Isolation of Mouse Embryos and PCR Genotyping**—Details concerning generation of conditional *Ada3* knock-out construct and *Ada3* knock-out mouse as well as PCR genotyping strategies are described in the supplemental data.

**Cell Culture Procedures and Viral Infections**—Embryonic day 13.5 embryos were dissected from *Ada3<sup>FL/+</sup>* intercrossed females, and MEFs were isolated and immortalized following the 3T3 protocol (23). MEFs were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Adenoviruses expressing EGFP-Cre or enhanced green fluorescent protein (EGFP) alone were purchased from the University of Iowa (Gene Transfer Vector Core). An adenovirus dose of 50–100 MOI diluted in 4 ml of serum-free medium was added to cells in 100-mm culture dishes (at about 30% confluence) and incubated for 1 h each at room temperature and at 37 °C followed by the addition of 7 ml of complete medium. After overnight incubation at 37 °C, medium was replaced with complete medium, and cells were carried further for various experiments. To generate retroviral FLAG-h*Ada3* vector, full-length FLAG-h*Ada3* (15) was cloned into pMSCVpuro vector (Clontech). Retroviruses were generated by transiently transfecting this retroviral construct into the Phoenix ecotropic packaging cell line using the calcium phosphate co-precipitation method. The retroviruses were transduced into *Ada3<sup>FL/FL</sup>*

MEFs by three infections at 12-h intervals using supernatant from transfected Phoenix cells to generate *Ada3<sup>FL/FL</sup>* MEFs expressing FLAG-h*Ada3*. Scrambled shRNA (5'-GGTTAAACCTTACGATGT-3') or p27 shRNA (5'-GTGGAATTCGACTTTCAG-3') was introduced into *Ada3<sup>FL/FL</sup>* MEFs by using three infections at 12-h intervals of the shRNA bearing pSUPER.retro.puro (Oligoengine) retrovirus containing supernatants from Phoenix cells. Retroviral infections were carried out in the presence of 8 μg/ml Polybrene (Sigma) and were followed by selection in 2 μg/ml puromycin for 48 h until complete loss of uninfected cells.

**Proliferation Assay, Colony Formation Efficiency Assay, and Cell Cycle Analysis**—To perform proliferation assays, 1 day after adenovirus infection, cells were plated at different numbers in 6-well plates in triplicates ( $5 \times 10^4$  (for counting on day 3),  $2.5 \times 10^4$  (for counting on day 5),  $1.25 \times 10^4$  (for counting on day 7), and  $0.625 \times 10^4$  (for counting on day 9) and counted at the indicated time points. For colony formation assay, cells 3 days after adenovirus-infection were trypsinized and plated at 1000 cells per 100-mm culture dishes in triplicates and carried for 15 more days with medium change as required. At the end of incubation, colonies in dishes were fixed and stained with crystal violet solution (0.25% crystal violet in 25% methanol) and photographed. For cell cycle analysis, 2 days after plating and adenoviral infection of  $2 \times 10^5$  cells in 100-mm culture dishes, cells were synchronized by replacing the complete medium with DMEM + 0.1% FCS and incubating for 72 h. Synchronized cells were stimulated with complete medium (DMEM + 10% FCS) for various time points and harvested and stained with propidium iodide (PI) for FACS analysis. For synchronization of cells at G<sub>2</sub>/M phase, 48 h after adenovirus infection, cells were switched to complete medium containing 125 ng/ml nocodazole for 18 h. Following synchronization, cells were washed three times with PBS and stimulated with complete medium for various time points and analyzed by FACS after PI staining.

**Generation of *Ada3* Monoclonal Antibody and Immunoblotting**—Antibodies used in this study can be found in the supplemental data.

**In Vitro Kinase Assay**—*In vitro* kinase assay was performed using purified histone H1 (Roche Applied Science) or Rb (769) (Santa Cruz Biotechnology sc-4112) as a substrate. Adenovirus-infected MEFs were starved for 3 days and stimulated with serum. Cells were harvested in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 0.1 mM Na<sub>4</sub>VO<sub>3</sub>, 1 mM NaF, and protease inhibitor mixture), and cyclin-dependent kinase (Cdk) complex was recovered by immunoprecipitation with 2 μg of either anti-Cdk4 (sc-56277)/Cdk6 (sc-53638) antibodies mixture or anti-Cdk2 (sc-6248) antibody (Santa Cruz Biotechnology). Cdk4/6 or Cdk2 complexes were captured with protein G-agarose for 1 h and washed with lysis buffer followed by one wash with kinase buffer (50 mM Tris-HCl (pH 7.5), 7.5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.1 mM Na<sub>4</sub>VO<sub>3</sub>, and 1 mM NaF). Cdk2 complex was incubated with histone H1 (2 μg) or Rb (500 ng), whereas Cdk4/6 complex was incubated with only Rb (500 ng) in kinase buffer containing 10 mM β-glycerophosphate, 33 μM ATP, and 10 μCi of [ $\gamma$ -<sup>32</sup>P]ATP (10 mCi/ml, 6000 Ci/mmol) at room temperature for 20 min. The products were

## Ada3 Regulates Cell Cycle Progression

subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes (PVDF), and autoradiographed.

**Analysis of the p27 Protein Turnover—Ada3<sup>FL/FL</sup> MEFs** were plated in 100-mm dishes and infected with control or Cre adenoviruses. For analyzing p27 protein half-life in exponentially growing cells, 2 days after adenovirus infection, cells were treated with 50  $\mu$ g/ml cycloheximide (Sigma) and harvested at the indicated time points. For analyzing p27 protein half-life in serum-starved cells, 2 days after adenovirus infection, cells were starved for 72 h in 0.1% serum-containing medium. Subsequently, 50  $\mu$ g/ml cycloheximide was added to the medium, and cells were harvested at the indicated time points. Total cell extracts were prepared, and equivalent amounts were run on SDS-PAGE and analyzed by Western blotting. Densitometry analysis was carried out on scanned images using ImageJ software.

**RNA Extraction and Quantitative Real-time PCR**—TRIzol reagent (Invitrogen) was used to isolate total RNA from MEFs infected with control virus or Cre adenovirus. 2  $\mu$ g of total RNA was used for reverse transcriptase reaction using SuperScript<sup>TM</sup> II reverse transcriptase (Invitrogen). Real-time PCR quantification was performed in triplicates using SYBR Green PCR master mix (Applied Biosystems) and the primers listed in supplemental Table S3. Expression levels were normalized against  $\beta$ -actin mRNA levels, and the results were calculated by the  $\Delta\Delta C_t$  method.

**Chromatin Immunoprecipitation Experiments**—Approximately 0.7 million *Ada3<sup>FL/FL</sup>* MEFs were plated in 100-mm dishes and infected with control or Cre adenoviruses. Forty-eight hours after infection, cells were synchronized with DMEM + 0.1% FCS for 72 h and then stimulated with complete medium (DMEM + 10% FCS) for 0–60 min as indicated for each experiment in Fig. 8C. ChIP experiment was performed by using the ChIP-IT Express kit from Active Motif. PCR amplification was performed using primers for the *c-myc* enhancer (forward, 5'-CTAGAACCAATGCACAGAGC-3'; reverse, 5'-CTCCCAGGACAAACCCAAGC-3') and for the *Skp2* promoter (forward, 5'-GCCATCGAGACCCCGGAGAT-3'; reverse, 5'-TGAGTCCCTTCCAGACGCTGT-3'). Control PCR was performed using primers for the *c-myc* distal site (forward, 5'-ACACACCTTGAATCCCGT-3'; reverse, 5'-CCCAGCTAGAATGAAGAAG-3') and the *Skp2* distal site (forward, 5'-GTGCTAGCTGCTTACCTTTGT-3'; reverse, 5'-GATAAGGATGCACTCTGGGGC-3'). PCR products were analyzed on 2% agarose/Tris-acetate-EDTA gels with ethidium bromide stain. PCR of the input DNA prior to immunoprecipitation was used as a control.

**Generation of Recombinant Baculoviruses and Ada3-His Expression Using Bac-to-Bac<sup>®</sup> Expression System**—*Ada3* baculoviral construct information and recombinant protein purification are detailed in the supplemental data.

**HAT Assay**—Protocol used for *in vitro* HAT assay can be found in the supplemental data.

**Microarray Analyses**—Protocol for microarray analyses is described in the supplemental data. The microarray data from this publication have been submitted to the GEO database and have been assigned the following Series record: GSE37542.

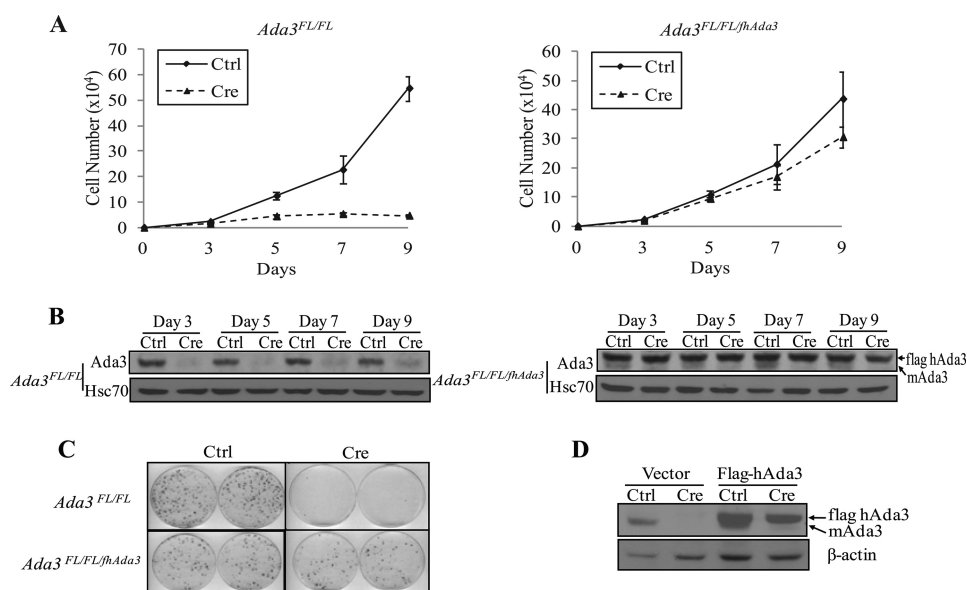
**TABLE 1**  
Genotype analysis of embryos from heterozygous intercrosses

Stage	Total no. of embryos	No. (%) of embryos			
		WT	Heterozygous	KO	Resorbed
Live born	224	75 (33)	149 (66)	0	0
E12.5	14	3 (21)	5 (36)	0	6 (43)
E 9.5	15	8 (53)	2 (13)	0	5 (33)
E 8.5	44	12 (27)	27 (61)	0	5 (11)
E 3.5	15	4 (27)	7 (47)	4 (27)	0

## RESULTS

**Deletion of Ada3 Leads to Early Embryonic Lethality in Mice**—The targeting construct generated using the recombineering technique (supplemental Fig. S1A; see supplemental Materials and Methods) was electroporated into an ES cell line derived from the 129/Ola strain of mice. Screening of resultant neomycin-resistant colonies yielded three correctly targeted clones (supplemental Fig. S1B). One positive clone was microinjected into blastocysts. The resulting chimeras transmitted the targeted allele to their progeny as verified by PCR. The neomycin cassette flanked by Frt recombination sites was removed by crossing the *Ada3*-targeted mice to FlpE recombinase transgenic mice (B6.Cg-Tg (ACTFLPe) 9205Dym/J; stock number 005703). Homozygous *Ada3<sup>FL/FL</sup>* mice were viable and fertile and exhibited no gross abnormalities when compared with *Ada3<sup>FL/+</sup>* or *Ada3<sup>+/+</sup>* controls. To achieve *Ada3* deletion, heterozygous *Ada3*-targeted mice (*Ada3<sup>FL/+</sup>* mice) were bred with transgenic mice expressing the adenovirus E1a promoter-driven Cre (B6.FVB-Tg (E1a-Cre) C5379Lmgd/J). E1a directs Cre expression in a wide range of tissues including germ cells. Heterozygous *Ada3*-targeted, Cre transgene-positive mice were crossed to C57BL/6J (wild-type) mice to generate heterozygous *Ada3*-deleted, Cre transgene-negative (*Ada3<sup>+/-</sup>*) mice. Heterozygous *Ada3<sup>+/-</sup>* mice of a mixed 129/Sv  $\times$  C57BL/6 background were viable and fertile, and their median life span of more than 18 months was comparable with that of their control littermates (data not shown). Heterozygous *Ada3<sup>+/-</sup>* mice were intercrossed to obtain homozygous *Ada3*-null mice. No *Ada3<sup>-/-</sup>* mice were observed among 224 live born pups screened (Table 1). The ratio of wild type to heterozygous offspring was 1:2, indicating that the loss of one *Ada3* allele does not lead to haploinsufficiency in mice.

To assess the specific period of developmental failure in the *Ada3* knock-out mice, embryos derived from *Ada3<sup>+/-</sup>* intercrosses were genotyped at different stages of gestation using a duplex PCR method (supplemental Fig. S1, C and D). Because no homozygous mutant embryos were recovered beyond embryonic day 8.5 (E8.5; Table 1), blastocysts were isolated at 3.5 days postcoitum and genotyped directly by PCR (supplemental Fig. S1E). When compared with blastocysts of *Ada3<sup>+/+</sup>* and *Ada3<sup>+/-</sup>* genotypes, *Ada3<sup>-/-</sup>* blastocysts that attached to culture dishes showed severe growth retardation of the trophoblast layer, and the inner cell mass was absent (supplemental Fig. S1F). PCR analysis revealed that  $\sim$ 25% of blastocysts analyzed were null for *Ada3* (Table 1). These results demonstrate that *Ada3* plays a critical role in early embryogenesis in mice. The failure of *Ada3<sup>-/-</sup>* embryos to remain viable beyond E3.5 suggests a potential role of *Ada3* in cell proliferation because



**FIGURE 1. Ablation of Ada3 causes proliferation defect in MEFs.** A, growth curves of *Ada3<sup>FL/FL</sup>* (left) and *Ada3<sup>FL/FL/hAda3</sup>* (right) MEFs after control adenovirus (Ctrl) or Cre adenovirus (Cre) infection. Data are mean  $\pm$  S.E. from three independent experiments performed in triplicates. B, Ada3 protein levels at different time points after Cre adenovirus infection. Note that reconstituted control cells express both mouse (*mAda3*; lower band) and human (FLAG hAda3; upper band) proteins, whereas only hAda3 is seen in Cre adenovirus-infected cells. C, colony formation assay. Crystal violet staining of the indicated cells infected with control virus or Cre adenovirus grown for 10 days is shown. D, Western blotting of lysates from C showing exogenous and endogenous Ada3.

extensive cellular proliferation occurs during this early stage of embryogenesis (see later sections).

*Ada3 Is Ubiquitously Expressed in Adult Mouse Tissues—* Embryonic lethality of *Ada3<sup>-/-</sup>* mice suggested a potential role of Ada3 in growth and development of many tissues. To examine whether Ada3 is expressed in adult tissues, we analyzed the relative levels of Ada3 protein expression in a range of adult mouse tissues. For this purpose, lysates from various tissues of 8-week-old wild-type mice were subjected to immunoblotting using an anti-Ada3 monoclonal antibody generated in our laboratory (see supplemental Materials and Methods). As seen in supplemental Fig. S2, Ada3 is ubiquitously expressed in all the tissues with higher levels seen in the mammary gland, lung, and thymus. These results suggest potentially ubiquitous functional roles of Ada3 and are consistent with embryonic lethal phenotype of its germline deletion.

*Conditional Ada3 Deletion in MEFs Leads to Proliferation Arrest—* Given the embryonic lethality as a result of *Ada3* deletion, we resorted to a cellular model of conditional *Ada3* deletion to investigate its roles at the cellular level. For this purpose, we generated *Ada3<sup>FL/FL</sup>* mice by interbreeding *Ada3<sup>FL/+</sup>* mice and established MEFs from these mice. Conditional *Ada3* deletion was obtained by infecting *Ada3<sup>FL/FL</sup>* MEFs with an adenovirus expressing the Cre recombinase (adeno-Cre), with adeno-GFP serving as a control. To assess the effects of Ada3 on cell proliferation, equal numbers of control- and adeno-Cre-infected MEFs were plated a day after adenovirus infection, and cells were counted at the indicated time points up to 9 days. Notably, *Ada3*-deleted MEFs exhibited a significantly slower rate of proliferation when compared with control MEFs (Fig. 1A, left). To confirm that the defect in cell proliferation was specifically due to depletion of Ada3, we generated *Ada3<sup>FL/FL/hAda3</sup>* MEFs by retrovirally introducing human

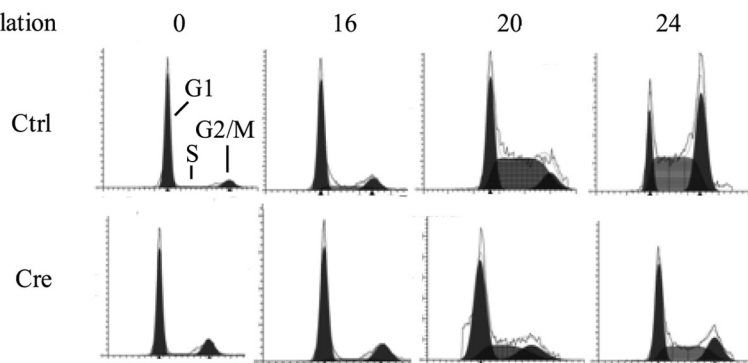
(hAda3) with an N-terminal FLAG tag into *Ada3<sup>FL/FL</sup>* MEFs. These transfectants were verified to be expressing the exogenous FLAG-tagged Ada3 protein (Fig. 1B). Similar to *Ada3<sup>FL/FL</sup>* MEFs, adeno-Cre infection of these cells led to deletion of endogenous *Ada3* and loss of its protein product (Fig. 1B). Notably, however, Cre-mediated deletion of *Ada3* in *Ada3<sup>FL/FL/hAda3</sup>* MEFs had a minimal effect on the proliferation of MEFs, whereas similar treatment of *Ada3<sup>FL/FL</sup>* MEFs led to reduction in the rate of proliferation; thus, the proliferative defect induced by deletion of mouse *Ada3* in MEFs was rescued by exogenous hAda3 (Fig. 1A, right). Colony formation efficiency assay, as an independent method to measure the extent of cell proliferation, further confirmed the proliferative defect of *Ada3*-deleted MEFs that could be rescued by reconstitution with exogenous hAda3 (Fig. 1, C and D).

*Ada3 Is Required for Cell Cycle Progression through G<sub>1</sub> to S Phase—* We reasoned that the proliferation defect upon *Ada3* deletion in MEFs could reflect a role of Ada3 in cell cycle progression. To directly examine whether Ada3 plays a role in cell cycle progression, *Ada3<sup>FL/FL</sup>* MEFs were infected with control and Cre adenoviruses, arrested in G<sub>0</sub>/G<sub>1</sub> by serum deprivation for 72 h, and then synchronously released into cell cycle by serum stimulation. FACS-based cell cycle analysis of propidium iodide-stained cells showed significant delay in G<sub>1</sub> to S progression in *Ada3*-deleted MEFs when compared with control MEFs (Fig. 2A). Of note, the relative distribution of S phase in *Ada3*-null MEFs after 20 h of serum stimulation was about half ( $31.6 \pm 2.33$  S.E. %) of the control virus-infected MEFs ( $56.05 \pm 4.71$  S.E. %) (Fig. 2B). These results demonstrate that conditional deletion of *Ada3* leads to delay in G<sub>1</sub> to S progression in MEFs, indicating an essential role of Ada3 in efficient G<sub>1</sub>/S progression.

## Ada3 Regulates Cell Cycle Progression

**A**

Hours after serum re-stimulation



**B**

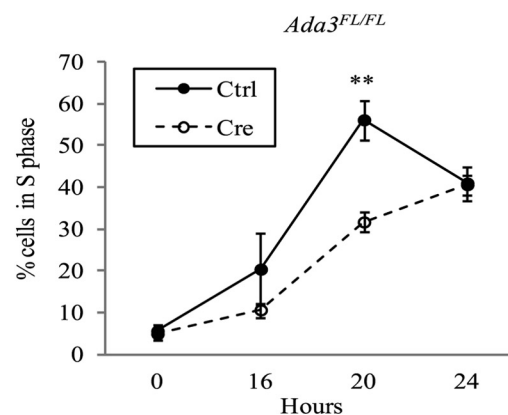


FIGURE 2. **Ada3** disruption delays  $G_1$  to  $S$  transition in MEFs. *A*, control (Ctrl)- or Cre- infected  $Ada3^{FL/FL}$  MEFs were serum-starved for 72 h and then released from synchrony as described under “Experimental Procedures” and processed for PI staining followed by FACS analysis. Cells in different phases of the cell cycle are shown from a representative experiment. *B*, graph derived from three independent experiments performed as in *A*, showing the proportion of cells entering into  $S$  phase at the indicated times after serum re-stimulation. Error bars are mean  $\pm$  S.E. from three independent experiments (\*\*,  $p = 0.0096$ , two-tailed Student’s  $t$  test).

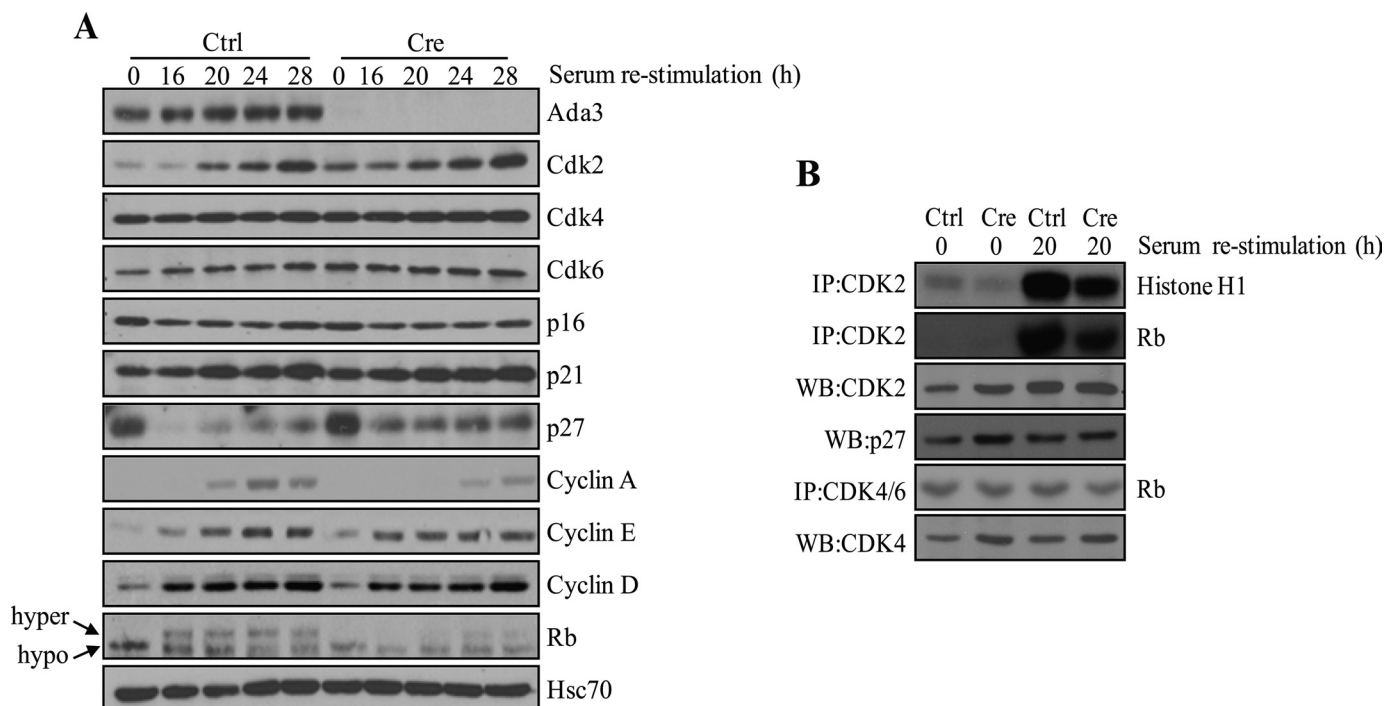
*Elevated p27<sup>Kip1</sup> Levels and Impaired Rb Phosphorylation upon Conditional Ada3 Deletion*—Given the delay in  $G_1$ /S progression imposed by induced *Ada3* deficiency, we examined the status of key proteins known to control the  $G_1$ /S transition. A well established and critical event during  $G_1$  to  $S$  progression is the phosphorylation of Rb by Cdk complexes (particularly complexes containing Cyclins D, E, or A), such as Cdk4/6 and Cdk2 (24, 25); phosphorylation of Rb leads to its release from Rb/E2F complexes, relieves E2Fs from repression, and facilitates the expression of E2F-responsive genes important for  $S$  phase progression (24, 25). Furthermore, degradation of Cdk inhibitors, such as p27, is required for progression of cells from  $G_1$  to  $S$  phase (26, 27). Therefore, we carried out Western blotting of cell lysates obtained from control *versus* conditional *Ada3*-deleted MEFs released into synchronous cell cycle progression to assess the levels of proteins relevant to the  $G_1$  to  $S$  phase transition. Notably, although minimal to no changes were observed in the levels of Cdk2, Cdk4, Cdk6, p16, p21, cyclin E, and cyclin D, a significant increase in p27 levels, a delay in the cell cycle-associated increase in cyclin A levels, and a lower level of Rb phosphorylation were observed in MEFs upon *Ada3* deletion when compared with control cells (Fig. 3A).

In view of increased levels of p27 without a significant change in the levels of Cdk proteins in cells with *Ada3* deletion, we assessed the level of Cdk2 kinase activity using an *in vitro* kinase assay on immunoprecipitates from cells. Although the Cdk4/6 kinase activity was comparable between control- and adeno-Cre-infected MEFs (Fig. 3B), the level of Cdk2 kinase activity was substantially reduced in Cre-infected MEFs when compared with control MEFs (Fig. 3B). These results suggest the potential reduction of Cdk2 kinase activity in the *Ada3*-deleted cells as a result of an increase in the levels of p27, accounting for defective Rb phosphorylation.

*Accumulation of p27 upon Ada3 Deletion Is due to Increased Stability of p27*—As accumulation of p27 levels upon *Ada3* deletion appeared to be functionally important, we examined whether this accumulation was at the transcriptional or post-transcriptional level. Real-time PCR analysis showed that

serum stimulation resulted in a marked reduction in the levels of *Cdkn1b* mRNA in both the control-infected and the Cre-infected cells (Fig. 4A); furthermore, the levels of *Cdkn1b* mRNA at various time points after serum addition remained comparable between the two cell populations, reinforcing the idea that the increase in p27 protein levels in *Ada3*-deleted cells was likely to be at a post-transcriptional level. As alterations in protein stability are a prominent mechanism to control Cdk inhibitor levels (28), we compared the half-life of p27 protein in WT *versus* *Ada3*-deleted MEFs using two distinct experimental formats; the first one utilized exponentially growing cultures, whereas the second one utilized cells first arrested in  $G_1$  by serum deprivation for 72 h followed by synchronous release into cell cycle by serum addition. In each case,  $Ada3^{FL/FL}$  MEFs infected with control or Cre adenoviruses were treated with cycloheximide to block new protein synthesis, and p27 levels in cell lysates following cycloheximide treatment were quantified using immunoblotting at various time points. Previous work has shown that p27 half-life in exponentially growing MEFs is about 3 h and increases to about 8 h in serum-starved cells (29). We found the p27 half-life in cells infected with control adenovirus was consistent with published results, *i.e.* approximately 2 h and 40 min in exponentially growing MEFs, whereas in growth-arrested cells, half-life was approximately 3 h and 30 min (Fig. 4, B–E). Notably, in both experimental formats, we observed a substantial increase in p27 protein half-life upon Cre-dependent *Ada3* deletion, with approximate half-lives of 4 h and 10 min and 6 h in exponentially growing *versus* synchronous culture formats, respectively. These results strongly support our conclusion that accumulation of p27 protein upon *Ada3* deletion is due to its increased stability.

*Depletion of p27 from Conditionally Deleted Ada3 MEFs Causes a Partial Rescue of G<sub>1</sub>/S Progression Defects*—Reduced activity of the p27 target Cdk2 in *Ada3*-deleted MEFs strongly suggested a role for p27 in defective cell cycle progression in these cells. To directly establish whether this is the case, we generated stable p27 knockdown  $Ada3^{FL/FL}$  MEFs ( $Ada3^{FL/FL/p27shRNA}$ ) by infecting  $Ada3^{FL/FL}$  MEFs with a retro-



**FIGURE 3. Effect of Ada3 depletion on expression of cell cycle regulator proteins and Cdk2 kinase activity.** *A*, *Ada3*<sup>FL/FL</sup> MEFs infected with control (*Ctrl*) and Cre adenoviruses serum-starved for 72 h, released from synchrony as described under "Experimental Procedures," and processed for immunoblot analysis of the indicated cell cycle proteins. *hyper*, hyperphosphorylated; *hypo*, hypophosphorylated. *B*, anti-Cdk2 or anti-Cdk4/6 immunoprecipitations performed using 300- $\mu$ g extracts of *Ada3*<sup>FL/FL</sup> MEFs infected with control or Cre adenovirus were subjected to *in vitro* kinase assay using histone H1 or Rb as a substrate. WB, Western blot; IP, immunoprecipitation.

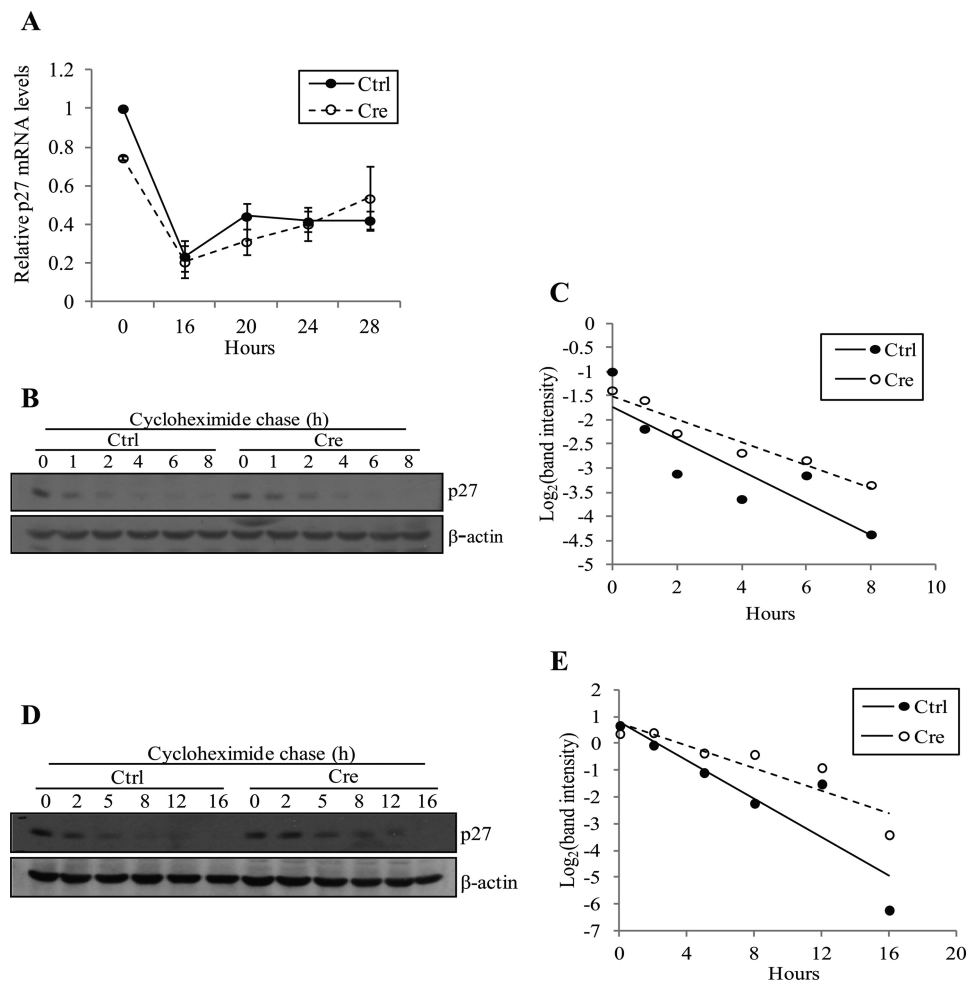
virus expressing a p27-specific shRNA followed by selection in puromycin, which resulted in a significant knockdown of p27 expression in these cells (Fig. 5A). Next, we infected the *Ada3*<sup>FL/FL/p27shRNA</sup> MEFs with control or Cre adenovirus and analyzed these for cell cycle progression using serum deprivation followed by serum stimulation, as above (Fig. 5B). Notably, a partial but clear rescue of the G<sub>1</sub>/S delay was observed in p27 shRNA-expressing cells, as seen by a much larger percentage of cells entering the S phase ( $41.4 \pm 3.5$  S.E. % in p27shRNA expressing conditionally deleted *Ada3* MEFs versus  $31.6 \pm 2.33$  S.E. % in *Ada3*-deleted MEFs at 20 h; compare Fig. 5C with Fig. 2B). Importantly, the levels of cyclin A, which is known to be expressed during G<sub>1</sub>/S transition and to peak in the S phase, as well as hyperphosphorylation of Rb, were essentially fully rescued by p27 shRNA knockdown (Fig. 5D; compare with Fig. 3A). Taken together, these results clearly demonstrate an important role of Ada3-dependent control of p27 levels in promoting cell cycle progression.

**Deletion of Ada3 Leads to Reduced Protein and mRNA Levels of Skp2 and c-Myc**—Given the causal link established above between p27 accumulation and G<sub>1</sub>/S cell cycle delay upon *Ada3* deletion, we wished to examine the molecular mechanism by which loss of Ada3 promotes p27 stability. Published studies have established a major role of Skp2-containing E3 ubiquitin ligases in regulating p27 protein turnover during cell cycle progression (30). As *Skp2* is a transcriptional target of c-Myc (31) and Ada3-containing STAGA complex has been shown to increase *myc* mRNA transcription (32, 33), the possibility of an Ada3-c-Myc-Skp2-p27 regulatory pathway appeared to be a plausible mechanism for our findings. To

explore this hypothesis, we first examined the effects of *Ada3* deletion on the levels of Skp2 mRNA (real-time PCR) and protein (immunoblotting). For this purpose, *Ada3*<sup>FL/FL</sup> cells infected with control or Cre adenovirus were serum-deprived and released into synchronous cell cycle progression by adding serum followed by analyses of Skp2 mRNA and protein at various time points. Notably, Skp2 mRNA and protein levels were substantially lower at each comparable time point in adeno-Cre-infected versus control MEFs (Fig. 6, A and B). These results indicate that *Ada3* deletion indeed leads to reduction in Skp2 levels and that this effect is likely due to reduced *Skp2* gene transcription.

Next, we asked whether *Ada3* deletion alters c-Myc mRNA levels and whether Ada3 directly binds to c-myc promoter. Indeed, analysis of control versus *Ada3*-deleted MEFs stimulated with serum to undergo cell cycle progression demonstrated that c-Myc mRNA as well as protein levels were significantly lower at each time point examined upon deletion of *Ada3* from cells (Fig. 6, C and D). Consistent with this, we observed lower occupancy of mouse *Skp2* promoter by c-Myc upon deletion of *Ada3*, which supports our results (supplemental Fig. S3). Finally, to establish that Ada3 indeed participates in the enhancement of *myc* gene transcription, we carried out ChIP analysis to assess whether Ada3 is recruited to c-myc enhancer during cell cycle progression. Indeed, a rapid recruitment of Ada3, as well as RNA polymerase II (used as positive control), to c-myc enhancer at -1.4 kb relative to transcription start site (but not to a distal site at -5 kb) was seen upon serum stimulation of MEFs (Fig. 6E). As expected, we did not detect any signals after immunoprecipitation with anti-Ada3 antibody in cells infected with adeno-Cre. These results therefore sup-

## Ada3 Regulates Cell Cycle Progression



**FIGURE 4. Deletion of *Ada3* does not affect p27 transcription but extends p27 protein half-life.** *A*, unaltered p27 mRNA levels after *Ada3* deletion. Real-time RT-PCR analysis of p27 mRNA levels from cells as treated in Fig. 2 was performed. Signals were normalized to  $\beta$ -actin levels and plotted relative to the level of p27 mRNA in starved control (*Ctrl*) cells. Error bars show mean  $\pm$  S.E. from three independent experiments. *B–E*, *Ada3* deletion in MEFs extends p27 half-life. *B*, 48 h after adenovirus infection, MEFs were treated with 50  $\mu$ g/ml cycloheximide and harvested at the indicated time points, and p27 and  $\beta$ -actin protein levels were analyzed by immunoblotting. *C*, the intensity of p27 bands was quantified by densitometry, normalized to  $\beta$ -actin using ImageJ software, and plotted against the time of cycloheximide treatment. Each decrease of 1 unit of log<sub>2</sub> is equivalent to one half-life. The lines were generated by linear regression formula. *D*, after 48 h of adenovirus infection, MEFs were starved using 0.1% serum-containing medium for 72 h and subsequently treated with 50  $\mu$ g/ml cycloheximide and harvested at the indicated time points. Cell lysates were analyzed by Western blotting using antibodies against p27 and  $\beta$ -actin. *E*, graph made from experiment in *D* by using the same procedure as in *C*.

port the existence of a novel cell cycle-associated, *Ada3*-regulated signaling pathway that promotes G<sub>1</sub>/S cell cycle progression by regulating p27 stability through Myc-dependent control of *Skp2* expression.

***Ada3* Deletion Leads to Decreased Histone Acetylation**—As we observed a partial rescue of G<sub>1</sub>/S transition in *Ada3*-deleted MEFs after knockdown of p27, we speculated that *Ada3* deletion-induced cell cycle arrest may involve other pathways as well. Given the known literature on *Ada3* as part of HAT complexes (8, 9), we examined whether *Ada3* is involved in controlling global histone acetylation. Therefore, we assessed the effect of *Ada3* deletion on lysine acetylation of various core histones. We expressed Cre recombinase in *Ada3*<sup>FL/FL</sup> MEFs and harvested protein samples from asynchronous cultures after 3 days of infection. Western blotting using antibodies against important acetylated lysine residues of all four core histones (H2A-K5, H2B-K5, H3-K9, H3-K56, and H4-K8) showed a significant reduction in acetylation at all these sites in *Ada3*-deficient MEFs when compared with

control MEFs (Fig. 7A), indicating that *Ada3* is essential in maintaining global histone acetylation.

We further examined the effect of *Ada3* deletion on acetylation of core histones after synchronizing cells in G<sub>1</sub> phase and subsequent release. There was a dramatic down-regulation of H3-K9 acetylation and a slight decrease in acetylation of H2B-K5 in *Ada3*-deleted MEFs when compared with control-MEFs, whereas this defect was rescued in *Ada3*<sup>FL/FL</sup> MEFs reconstituted with exogenous human FLAG-*Ada3* (Fig. 7B), suggesting that the defect in histone acetylation seen in *Ada3*-deleted MEFs was a consequence of *Ada3* deletion. Histone acetylation has been shown to be important for deposition of histones during replication-coupled nucleosome assembly as well as for chromatin maturation following DNA replication (34, 35). Thus, the partial rescue in G<sub>1</sub> to S transition observed upon knockdown of p27 in *Ada3*-deficient cells could be attributed to massive histone acetylation defects, which would create difficulties for cells to undergo DNA replication and thus delay transition through S phase.

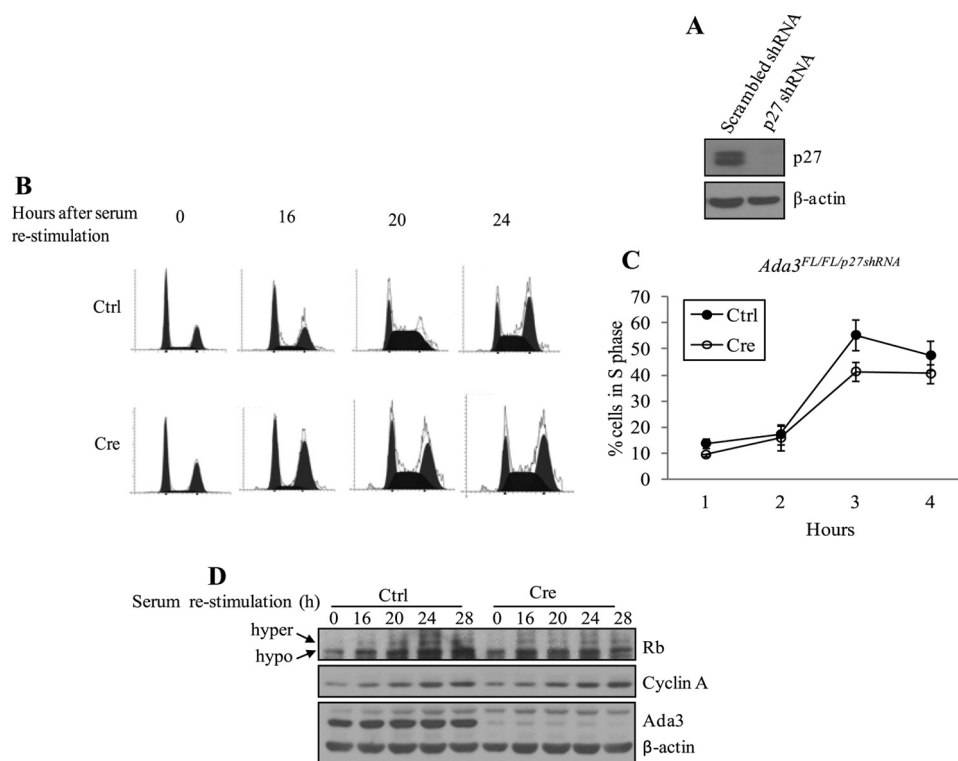


FIGURE 5. **p27 depletion partially rescues G<sub>1</sub> to S transition defects seen in *Ada3*-null MEFs.** *A*, *Ada3*<sup>FL/FL</sup> MEFs were infected with retrovirus-expressing scrambled or p27 shRNA followed by selection for 2 days in puromycin and analyzed by immunoblotting using p27 and β-actin antibodies. *B*, PI staining and FACS analysis of *Ada3*<sup>FL/FL</sup> MEFs expressing p27 shRNA that were infected with either control (*Ctrl*) or Cre adenoviruses and synchronized as in Fig. 2. *C*, graph derived from three experiments as in *B* showing the proportion of cells entering into S phase at the indicated times after serum restimulation. *Error bars* indicate mean ± S.E. from three independent experiments. *D*, immunoblotting of protein samples from *B* showing rescue of hyperphosphorylated (*hyper*) Rb and cyclin A levels. *hypo*, hypophosphorylated.

*Recombinant Ada3 Stabilizes HAT Enzymes and Enhances Their Activity*—Ada3 protein has been identified as an important component of protein complexes containing HAT enzymes. Therefore, we subjected samples harvested after 3 days of *Ada3* deletion to immunoblotting with two important HATs such as p300 and PCAF. Indeed, deletion of *Ada3* caused drastic down-regulation of p300 and PCAF in MEFs (Fig. 7C). Notably, *Ada3* deletion had no effect on the mRNA levels of p300 and PCAF (data not shown). Thus, the defects in histone acetylation seen in *Ada3*-null MEFs could be attributed to the effect of *Ada3* deletion on stability of important HATs in cells.

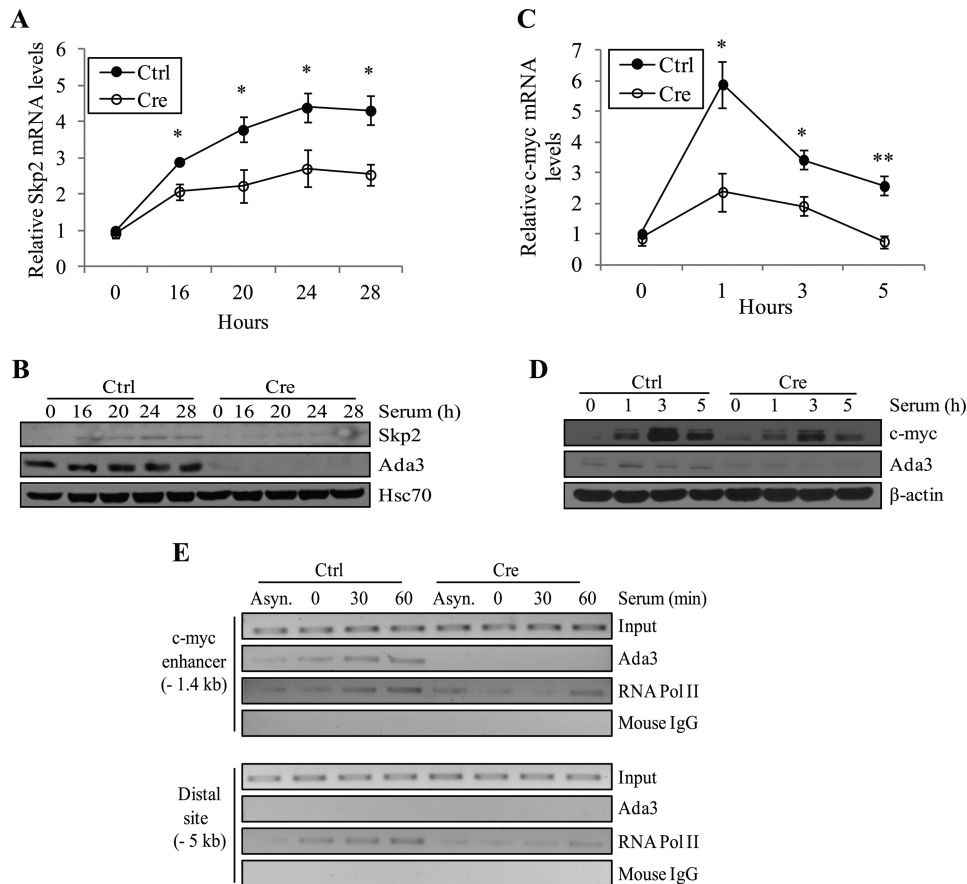
In addition to the role of *Ada3* in stability of HAT enzymes, we explored whether *Ada3* catalyzes the activity of HAT enzymes. Although *Ada3* is shown to be important in maintaining stability of HAT complexes, it has not been demonstrated whether *Ada3* directly modulates the activity of known HAT enzymes such as p300. Thus, we expressed and purified baculoviral hAda3 and used it in an *in vitro* assay in which HAT activity of p300 histone acetyl transferase enzyme on histone substrates was measured. As seen in Fig. 7D, increasing amounts of *Ada3* resulted in increased acetylation of histone H1 and histone H3 by p300, suggesting that *Ada3* plays an important role in enhancing the HAT activity of p300. To further explore the role of *Ada3* in histone acetylation, we used only histone H3 as a substrate and observed an *Ada3* dose-dependent increase in acetylation of histone H3 by p300 (Fig. 7E). Thus, *Ada3* manifests its effect on histone acetylation by main-

taining the integrity of various HAT complexes and by enhancing the catalytic activity of HATs.

*Deletion of Ada3 Leads to Global Gene Expression Changes*—Given the links between *Ada3* and transcriptional activation, we used control and *Ada3*-deleted cells to perform microarray analyses. As expected, the expression of multiple genes was altered; 539 genes were down-regulated and 928 genes were up-regulated ≥ 1.5-fold upon *Ada3* deletion (supplemental Table S1). Validation of some of the deregulated genes from microarray by real-time PCR showed good co-relation with the microarray data (supplemental Fig. S4). Ingenuity pathway analyses showed that most of the genes affected were involved in controlling cell growth, proliferation, and cell death (supplemental Table S2, top biological functions affected; cell growth and proliferation (386 genes) and cell death (359 genes)). The top network affected was the RNA post-transcriptional modification and cellular assembly and organization network, whereas the cell cycle, endocrine system development and function, and cancer network was the third most affected network (supplemental Fig. S5). Notably, *c-myc* and *Skp2* genes that we described above were down-regulated 1.4- and 1.43-fold, respectively. This is lower than what we observed by real-time PCR and could be attributed to the fact that microarray data were performed on asynchronous populations, whereas the real-time PCR data were performed on synchronous cells (Fig. 6, A and C). Interestingly, many of the genes present in cell growth and proliferation set were those involved in controlling cell division as well as some involved in DNA replication (Table 2).



## Ada3 Regulates Cell Cycle Progression



**FIGURE 6. Deletion of *Ada3* from MEFs leads to reduced mRNA and protein levels of *Skp2* and *c-Myc*.** *A*, analysis of *Skp2* mRNA levels by real-time RT-PCR from cells as treated in Fig. 2. Signals were normalized to  $\beta$ -actin levels and plotted relative to the level of *Skp2* mRNA in starved control cells. *Error bars* represent mean  $\pm$  S.E. from three independent experiments (\*,  $p = 0.015, 0.036, 0.043,$  and  $0.032$  for 16, 20, 24, and 28 h, respectively by two-tailed Student's *t* test). *B*, immunoblots showing *Skp2* protein levels in cells treated as in *A*. *C*, analysis of *c-Myc* mRNA levels by real-time RT-PCR from cells as treated in Fig. 5. Signals were normalized to  $\beta$ -actin levels and plotted as in *A*. *Error bars* show mean  $\pm$  S.E. from three independent experiments. *D*, immunoblots showing *c-Myc* protein levels in cells treated as in *C* (\*,  $p = 0.023$  and  $0.027$  for 1 and 3 h, respectively; \*\*,  $p = 0.008$  by two-tailed Student's *t* test). *E*, occupancy of *Ada3* on the *c-myc* enhancer. Chromatin fragments from control (*Ctrl*) and *Cre Ada3<sup>FL/FL</sup>* MEFs cells were immunoprecipitated with anti-*Ada3* antibody. Chromatin fragments were prepared from Asynchronous (*Asyn.*) cells as well as from cells synchronized with 0.1% serum containing DMEM for 72 h (*lane 0*) and stimulated with serum with indicated time points. The immunoprecipitated DNA was analyzed by PCR, using *c-Myc* enhancer-specific primers. Primers amplifying a region that is 5 kb upstream of the *c-Myc* enhancer were used as a negative control. *RNA Pol II*, RNA polymerase II.

*Ada3* Deletion Leads to Defects in Cell Division and Accumulation of Abnormal Nuclei—Based on our microarray analyses where several mitotic genes were affected upon deletion of *Ada3* and a recent study showing the role of *Ada3* in mitosis upon shRNA deletion (14), we examined the effect of *Ada3* deletion on mitotic phase of cell cycle. These analyses showed that *Cre*-mediated *Ada3* deletion led to increased accumulation of cells with abnormal nuclei when compared with control MEFs. *Ada3*-deficient MEFs showed various nuclear abnormalities such as fragmentation, lobulation, and multinucleation (Fig. 8A). When compared with  $13.08 \pm 2.39$  S.E. % control MEFs,  $83.41 \pm 3.45$  S.E. % of *Ada3*-deficient MEFs showed abnormal nuclei (Fig. 8B). Live imaging of cells for 24 h showed that the majority of *Ada3*-deleted cells failed to divide normally. Some of the cells snapped back while attempting to undergo cytokinesis, leading to the formation of binucleated cells, whereas other cells that had normal nucleus before mitosis showed fragmented nuclei afterward and were unable to divide. In other cases, cell division resulted in the formation of anucleated daughter cells (Representative images shown

in supplemental Fig. S6). Taken together, these results demonstrate an indispensable role of *Ada3* in normal cell cycle progression. The cell division defect results reported here corroborate with an earlier published study showing similar defects upon shRNA knockdown of *Ada3* (14). Mitotic defects observed in their study were attributed to acetylation of a non-histone substrate cyclin A, and no changes in histone acetylation upon knockdown of *Ada3* were reported. In contrast, we observed a dramatic change in global histone acetylation and expression of various genes involved in mitosis. Although at present we cannot explain this discrepancy, the differences in the results may be partly attributable to the use of different cellular systems and differences in approaches followed such as shRNA or *Cre*-mediated to delete *Ada3*.

*Deletion of *Ada3* Leads to Delay in  $G_2/M$  to  $G_1$  Progression*—As deletion of *Ada3* in MEFs led to defects in cell division, we reasoned that the disruption of *Ada3* should exert an effect on  $G_2/M$  to  $G_1$  transition. To examine this effect, we synchronized control- and *Cre*-adenovirus-infected *Ada3<sup>FL/FL</sup>* MEFs at

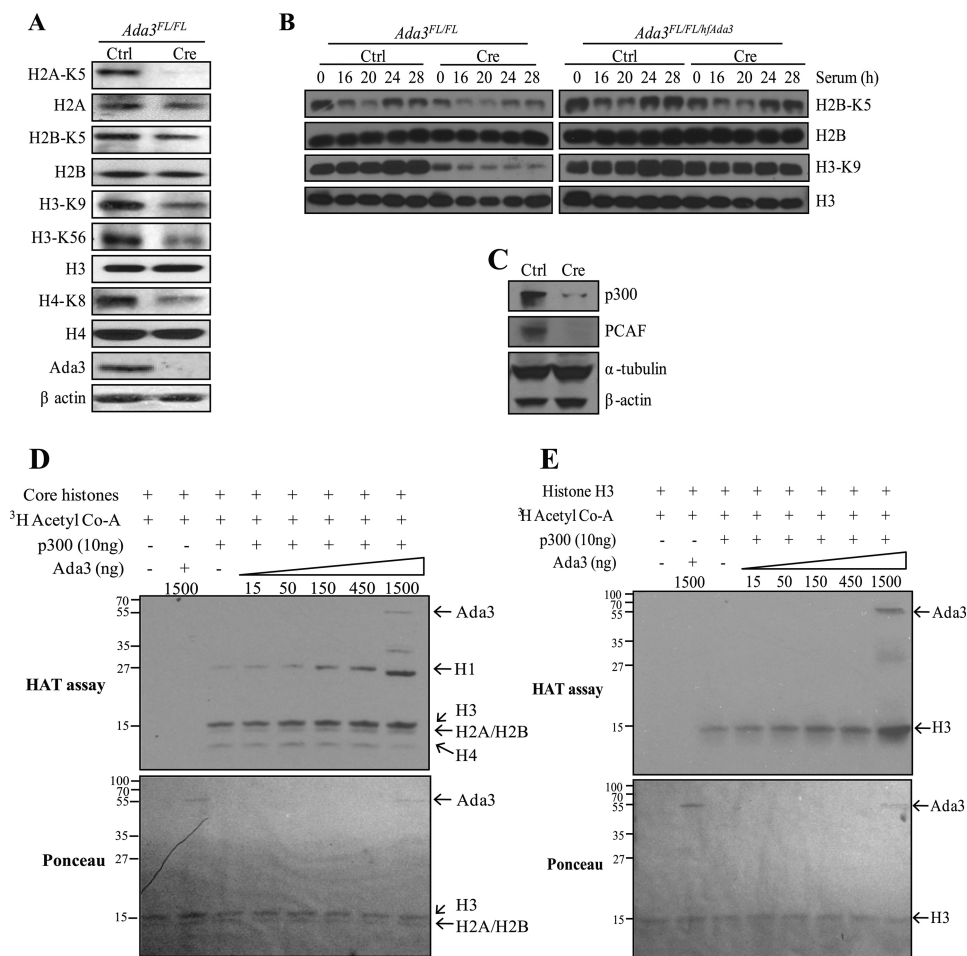


FIGURE 7. **Ada3 deletion abrogates histone acetylation by destabilizing various HATs.** A–C, Western blotting analysis of lysates from asynchronous (A and C) or serum-restimulated (B) *Ada3<sup>FL/FL</sup>* or *Ada3<sup>FL/FL</sup>/hAda3* MEFs infected with control (Ctrl) or Cre adenoviruses using the indicated antibodies. D and E, Ada3 enhances p300 HAT activity. *In vitro* HAT assay using purified recombinant human Ada3 and core histones (D) or histone H3 alone (E) along with their respective Ponceau blots to indicate equal loading is shown.

G<sub>2</sub>/M checkpoint by treating them with nocodazole and released them from synchrony followed by cell cycle analysis using flow cytometry (Fig. 8C). Nocodazole-synchronized *Ada3*-deleted MEFs showed a lower percentage of cells in G<sub>2</sub>/M phase (61%) at the 0-h time point when compared with control MEFs (80%) (Fig. 5C). On the contrary, we observed a higher percentage (20%) of *Ada3*-deleted MEFs in G<sub>1</sub> phase when compared with control MEFs (7%) after synchronization. We speculate that *Ada3*-deficient MEFs that are exhibiting a delay in G<sub>1</sub> to S transition were unable to get completely synchronized at G<sub>2</sub>/M checkpoint as these cells are potentially moving slowly through the G<sub>1</sub> to S transition and require a prolonged treatment with nocodazole to show a complete synchronization as seen in control MEFs. When we compared the percentage of cells moving into G<sub>1</sub> phase on release from nocodazole treatment in both *Ada3*-deficient and control MEFs, a significant impairment in G<sub>2</sub>/M to G<sub>1</sub> transition in *Ada3*-deleted MEFs was observed (Fig. 8D). Taken together, these results demonstrate a critical role of Ada3 in both G<sub>1</sub> to S transition as well as G<sub>2</sub>/M to G<sub>1</sub> transition in MEFs, indicating that the cell proliferation defect observed in *Ada3*-deficient MEFs is due to a combined defect in G<sub>1</sub> to S as well as G<sub>2</sub>/M to G<sub>1</sub> transition.

## DISCUSSION

Regulated cell cycle entry and progression are essential for precise developmental programs as well as to maintain organ homeostasis in adult animals. Although the basic components of cell cycle have been largely defined, regulatory control mechanisms that ensure orderly proliferative responses to physiological cues and whose aberrations underlie the vast instances of altered proliferation in cancer continue to be elucidated. We previously identified the ADA complex component Ada3 as a human papillomavirus E6 oncoprotein partner as well as a coactivator of cell cycle checkpoint regulator and tumor suppressor p53 (15, 20). Several *in vitro* studies have shown that Ada3 is an essentially universal component of a multitude of HAT-based transcriptional regulatory complexes (8, 9), and it has become essential to define its physiological roles using *in vivo* animal models.

Here, we demonstrate that Ada3 is essential for embryonic development in mice and that *Ada3*-null embryos undergo very early lethality. As an essential component of the transcriptional coactivator complexes that include HATs and promote histone acetylation of key gene targets, Ada3 is known to be essential for growth in yeast (16) as well as in model metazoan organisms

## Ada3 Regulates Cell Cycle Progression

**TABLE 2**

**List of deregulated genes involved in cell division and DNA replication**

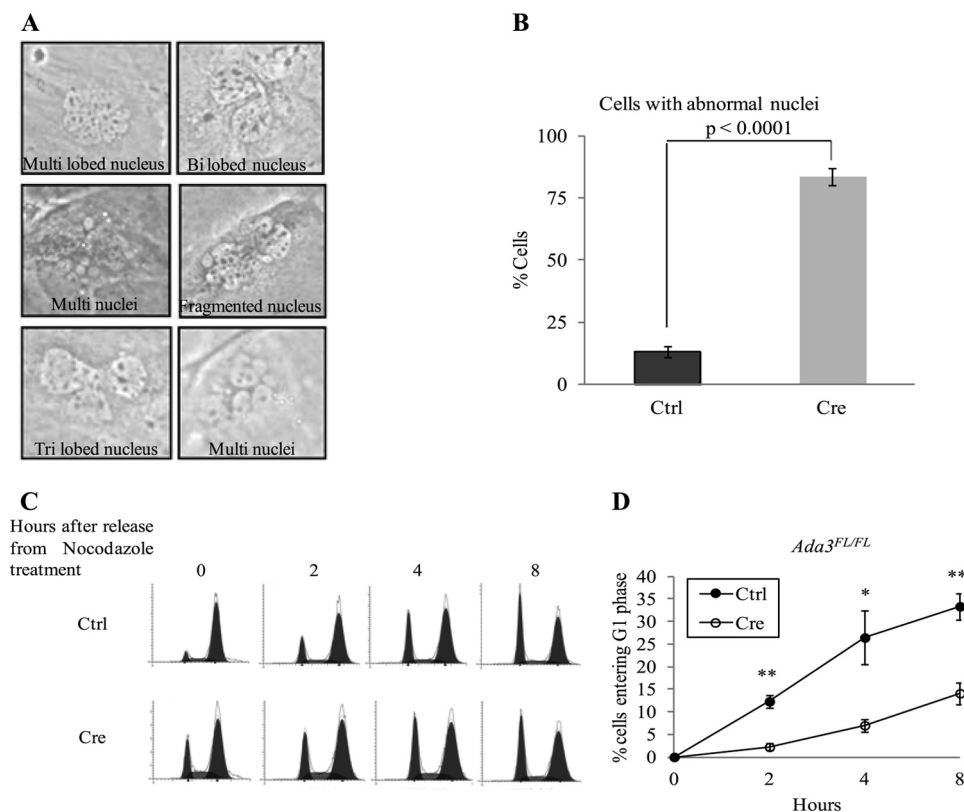
Genes down-regulated at least 1.5-fold upon loss of Ada3 as obtained from microarray analyses. The genes were classified based upon gene ontology biological processes.

Gene symbol	Gene title	-Fold down-regulated
<b>Genes involved in cell division</b>		
<i>Kifc1</i>	Kinesin family member C1, similar to Kifc1 protein	2.0
<i>Nfkbil1</i>	Nuclear factor of [kappa] light polypeptide gene enhancer in B-cells inhibitor-like 1	2.0
<i>Fbxo5</i>	F-box protein 5	1.8
<i>Cenpf</i>	Centromere protein F	1.8
<i>Cdc6</i>	Cell division cycle 6 homolog ( <i>Saccharomyces cerevisiae</i> )	1.7
<i>Kntc1</i>	Kinetochore-associated 1	1.7
<i>Baz1b</i>	Bromodomain adjacent to zinc finger domain, 1B	1.6
<i>Mlf1ip</i>	Myeloid leukemia factor 1 interacting protein	1.6
<i>Myh10</i>	Myosin, heavy polypeptide 10, non-muscle	1.6
<i>Kif11</i>	Kinesin family member 11	1.6
<i>Ccna2</i>	Cyclin A2	1.6
<i>Smc2</i>	Structural maintenance of chromosomes 2	1.6
<i>Plk1</i>	Polo-like kinase 1 ( <i>Drosophila</i> )	1.5
<i>Bub1b</i>	Budding uninhibited by benzimidazoles 1 homolog, $\beta$ ( <i>S. cerevisiae</i> )	1.5
<i>Aspm</i>	asp (abnormal spindle)-like, microcephaly-associated ( <i>Drosophila</i> )	1.5
<i>Anln</i>	Anillin, actin-binding protein	1.5
<i>Zwilch</i>	Zwilch, kinetochore-associated, homolog ( <i>Drosophila</i> )	1.5
<i>Mki67</i>	Antigen identified by monoclonal antibody Ki 67	1.5
<i>Mad2l1</i>	MAD2 mitotic arrest deficient-like 1 (yeast)	1.5
<i>Smc4</i>	Structural maintenance of chromosomes 4	1.5
<i>Cdca8</i>	Cell division cycle-associated 8	1.5
<i>Kif20b</i>	Kinesin family member 20B	1.5
<i>Hells</i>	Helicase, lymphoid-specific	1.5
<i>Ccnb1</i>	Cyclin B1	1.5
<i>Cdca3</i>	Cell division cycle-associated 3	1.5
<i>Nuf2</i>	NUF2, NDC80 kinetochore complex component, homolog ( <i>S. cerevisiae</i> )	1.5
<i>Ndc80</i>	NDC80 homolog, kinetochore complex component ( <i>S. cerevisiae</i> )	1.5
<i>Birc5</i>	Baculoviral IAP repeat-containing 5	1.5
<i>Bub1</i>	Budding uninhibited by benzimidazoles 1 homolog ( <i>S. cerevisiae</i> )	1.5
<i>Suv39h2</i>	Suppressor of variegation 3–9 homolog 2 ( <i>Drosophila</i> )	1.5
<i>Aurkb</i>	Aurora kinase B	1.5
<i>Wee1</i>	WEE 1 homolog 1 ( <i>Schizosaccharomyces pombe</i> )	1.5
<b>Genes involved in DNA replication</b>		
<i>Kitl</i>	Kit ligand	1.9
<i>Prim1</i>	DNA primase, p49 subunit	1.7
<i>Mcm7</i>	Minichromosome maintenance-deficient 7 ( <i>S. cerevisiae</i> )	1.7
<i>Ccne2</i>	Cyclin E2	1.7
<i>Pola1</i>	Polymerase (DNA directed), alpha 1	1.7
<i>Dtl</i>	Denticleless homolog ( <i>Drosophila</i> )	1.7
<i>Cdc6</i>	Cell division cycle 6 homolog ( <i>S. cerevisiae</i> )	1.7
<i>Ctf18</i>	CTF18, chromosome transmission fidelity factor 18 homolog ( <i>S. cerevisiae</i> )	1.7
<i>Nfib</i>	nuclear factor I/B	1.6
<i>Prim1</i>	DNA primase, p49 subunit	1.6
<i>Orc11</i>	Origin recognition complex, subunit 1-like ( <i>S. cerevisiae</i> )	1.6
<i>Rrm1</i>	Ribonucleotide reductase M1	1.6
<i>Rpa1</i>	Replication protein A1	1.6
<i>Cdt1</i>	Chromatin licensing and DNA replication factor 1	1.6
<i>Gins2</i>	GIN5 complex subunit 2 (Psf2 homolog)	1.5
<i>Rbbp4</i>	Retinoblastoma-binding protein 4	1.5
<i>Chaf1b</i>	Chromatin assembly factor 1, subunit B (p60)	1.5
<i>Tk1</i>	Thymidine kinase 1	1.5

such as *Drosophila* where Ada3 deficiency is associated with arrest in early development (36). However, this study is the first direct demonstration of an essential role of Ada3 in mammalian embryonic development. Notably, the embryonic developmental block imposed by *Ada3* deletion occurs very early, resulting in arrest of development at the blastocyst stage, the stage of embryonic development at which extensive cell proliferation occurs (37). Notably, studies that employed gene knockouts of subunits of several chromatin-modifying complexes, including *Gcn5*, *Trrap*, *Ep300*, *CBP*, *Hdac3*, or *Atac2*, also lead to early embryonic lethality (34, 38–42), consistent with an essential role of chromatin modification machinery in mammalian growth and development. However, except for *Trrap* knockout, which produces lethality at the blastocyst stage (42), knockouts of other genes produce embryonic developmental arrest at much later stages: for example, *Gcn5* (E9.5–E11.5), *Ep300* (E9.5–E10.5), and *Atac2* (E11.5) in comparison with

E3.5 block observed in *Ada3*-null mice. The relatively early developmental arrest of *Ada3*-null mice when compared with other regulators could reflect the role of Ada3 as a component of multiple chromatin-remodeling complexes (see Introduction and below). The distinct times of arrest seen with *Gcn5*-null and *Ada3*-null embryos are somewhat surprising and suggest the possibility that Ada3 may mediate early developmental roles through complexes in which *Gcn5* is not a critical component or is functionally redundant with other HATs. Consistent with this hypothesis, we observed that *Ada3*-deleted cells exhibit defects in multiple histone acetylations and show decrease in the levels of PCAF and p300 proteins.

We used the conditional deletion feature of the mouse model to assess the critical functional roles of Ada3 by utilizing Cre-dependent gene deletion in MEFs from *Ada3<sup>FL/FL</sup>* mice. This system provided a clear evidence that Ada3 plays an essential



**FIGURE 8. Abnormal cell division and delayed  $G_2/M$  to  $G_1$  transition in *Ada3*-deleted cells.** *A*, images of *Ada3<sup>FL/FL</sup>* cells after 5 days of infection with Cre adenovirus showing abnormal (fragmented, lobulated, or multi) nuclei. *B*, quantification of abnormal nuclei from cells infected with control (*Ctrl*) or Cre adenovirus; 5 days after infection, cells were fixed and stained with Giemsa stain and scored for abnormal nuclei (at least 100 cells from each group were counted). *Error bars* show mean  $\pm$  S.E. from three independent experiments. *C*, control- and Cre adenovirus-infected MEFs were treated for 20 h with nocodazole and were harvested at the indicated time points after release, stained with PI, and subjected to FACS analysis. *D*, graph showing the percentage of cells entering  $G_1$  phase after release from nocodazole treatment at various time points from experiments as in *C*. *Error bars* are mean  $\pm$  S.E. from three independent experiments (\*,  $p = 0.034$ ; \*\*,  $p = 0.0038$  and  $0.007$  for 4 and 8 h, respectively, by two tailed Student's *t* test).

role in cell proliferation by promoting  $G_1$  to S as well as  $G_2/M$  to  $G_1$  cell cycle progression. Furthermore, the proliferative arrest imposed by conditional deletion of *Ada3* was reversed by ectopic expression of human *Ada3*, indicating that the loss of *Ada3* itself, rather than alteration of any neighboring gene product, was responsible for the observed cell cycle phenotype.

Cell cycle progression is a tightly regulated process and is dependent on sequential and stringently controlled, concerted activation of Cdks and their inhibition by Cdk inhibitors. The novel cell cycle regulatory pathway downstream of *Ada3* was suggested by our initial analyses of alterations in the levels of core components of mammalian cell cycle machinery. These analyses revealed a dramatic reduction in the key propeller of  $G_1/S$  phase transition, hypophosphorylated Rb when *Ada3* was deleted. Association of this defect with reduced *Cdk2* activity without a reduction in *Cdk2* levels suggested the role of elevated p27, which we established directly by demonstrating that shRNA knockdown of p27 substantially alleviated the  $G_1/S$  block imposed by *Ada3* deficiency. Further biochemical connections were suggested by recent findings that STAGA complex, which includes *Ada3* as a component, enhances *c-myc* transcription (32, 33). Because *c-Myc* is shown to regulate the transcription of *Skp2*, an essential component of the SCF(*Skp2*) cell cycle-associated E3 ligase that regulates p27 levels, we sought and established evidence that cell cycle-associated Myc

transcription is *Ada3*-dependent and that *Ada3* is required for *Skp2* transcription (which is a downstream target of *Myc*) and p27 stability (regulated by SCF(*Skp2*)). We provided direct evidence for key elements of this model, including ChIP analyses that demonstrated the cell cycle-associated early recruitment of *Ada3* to *c-myc* enhancer elements. This result is consistent with independent findings from two groups that STAGA complex is recruited to *c-Myc* enhancer and regulates *c-myc* transcription (32, 33). In addition to control of *c-myc* gene transcription by *Ada3*-containing STAGA complex, studies have shown that STAGA associates with *c-Myc* on *c-Myc* target gene promoters and is required for efficient transcription activation by *c-Myc* (43, 44). This provides an additional mechanism by which *Ada3* could control *c-Myc*-driven target genes that regulate cell proliferation. Thus, *Ada3* might be involved in controlling both *c-myc* transcription as well as *c-Myc* function. Consistent with our observations, it is noteworthy that *c-myc* knock-out mice are embryonic lethal (45). Defective regulation of *c-Myc* transcription by *Ada3*-containing (STAGA or other) complexes might contribute to the early embryonic lethality seen in *Ada3*-null mice; further analyses of Myc-dependent pathways upon germline or conditional deletion of *Ada3* during embryogenesis should help establish whether this is the case.

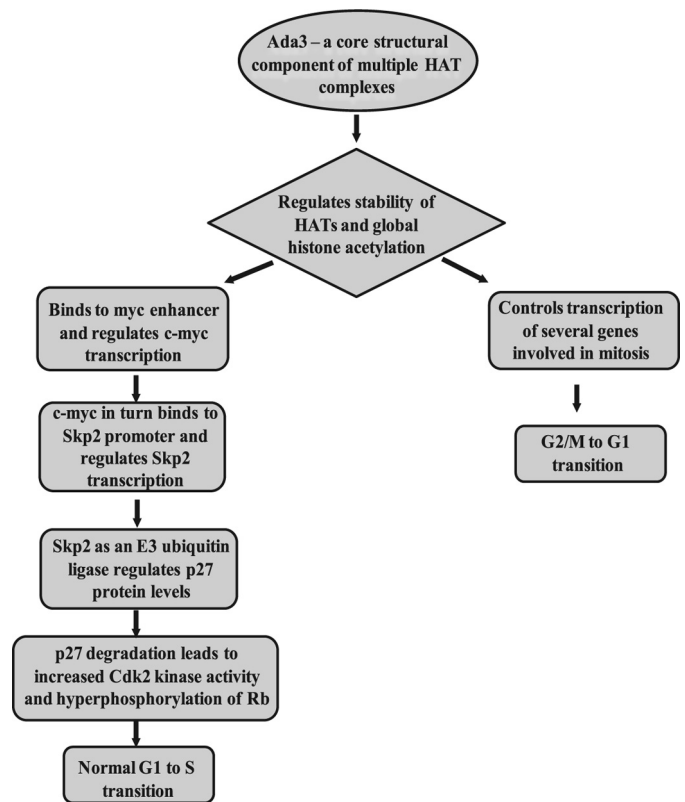
Although regulation of p27 protein stability by *Ada3* through control of *c-myc* transcription forms an important basis for

## Ada3 Regulates Cell Cycle Progression

G<sub>1</sub>/S transition defects, we were not able to fully rescue the defect in cell cycle by using p27 shRNA, suggesting the involvement of other cellular pathways. To this end, examining global histone acetylations in *Ada3*-deficient cells revealed dramatic defects in histone acetylation. Because *Ada3* forms a core structural component of various different HAT complexes in the cell, the presence of *Ada3* is highly essential for structural maintenance and proper functioning of these complexes in cells. Additionally, loss of *Ada3* led to substantial depletion of important HATs, p300, and PCAF proteins but not mRNA, which further explains the profound defects in histone acetylation seen upon loss of *Ada3*. This is consistent with the fact that PCAF and p300 are present in *Ada3*-containing protein complexes (8–11). These defects in histone acetylation could explain the partial rescue upon knockdown of p27 as histone acetylation has been shown to have an important role in the process of DNA replication (34, 35).

Given the role of *Ada3* in regulating global histone acetylation and that histone acetylation is important in transcriptional activation of genes, we performed microarray analysis and showed that several genes were deregulated upon *Ada3* deletion. Analysis of these genes by ingenuity pathway analysis revealed the RNA post-transcriptional modification and cellular assembly and organization network as the top affected network, with the cell cycle, endocrine system development and function, and cancer network as the third most affected. The top network affected in the microarray data is consistent with an earlier study, which showed that *Ada3*-containing STAGA complex interacts with pre-mRNA splicing machinery, components suggesting a role for this complex in mRNA splicing (46). Importantly, the top biological functions affected upon deletion of *Ada3* included those involved in cell growth and proliferation with 386 deregulated genes involved in this process. Thus, our microarray data confirmed a role of *Ada3* in cell cycle progression. Additionally, some of the top physiological functions affected upon deletion of *Ada3* were those involving tissue development and organismal survival (supplemental Table S2), which could be linked to the early embryonic lethality observed upon knock-out of *Ada3* in mouse.

Notably, many of the genes that were involved in regulating cell growth and proliferation were those involved in mitosis and some that were involved in DNA replication. This led us to examine cell division upon deletion of *Ada3*. Consistent with the microarray data, we observed massive nuclear abnormalities, cell division defects, and delay in G<sub>2</sub>/M to G<sub>1</sub> phase progression upon deletion of *Ada3*. Our observed phenomenon of cell division defects upon deletion of *Ada3* is consistent with a recently published study (14). The authors showed that ATAC HAT complex is specifically involved in regulating mitosis and that shRNA-mediated knockdown of *Ada3* or *Ada2a* led to defects in cell division, which were attributed to stabilization of cyclin A upon disruption of ATAC complex. Although we did not observe an increase in cyclin A levels (in fact the converse) in our system, we did observe a similar effect on nuclear abnormalities and a clear defect in mitosis. Furthermore, the authors did not observe any changes in histone acetylation defects upon depletion of *Ada3*, which is not consistent with our results. Of note, *Ada2a* is a component of only ATAC complex; however,



**FIGURE 9. Proposed model for the role of *Ada3* in cell cycle progression.** As a core structural component of various HAT complexes, *Ada3* maintains the integrity of HAT complexes and thus regulates global histone acetylation. *Ada3* regulates G<sub>1</sub> to S transition by controlling transcription of *c-myc* gene, which in turn controls *Skp2* gene expression by binding to its promoter. *Skp2* as an E3 ubiquitin ligase causes timely degradation of p27 protein so that cells can enter into S phase by increasing Cdk2 kinase activity, thus inducing hyperphosphorylation of Rb and cell progression from G<sub>1</sub> to S phase of cell cycle. Additionally, through controlling global histone acetylation, *Ada3* controls transcription of various genes involved in cell division and is required for cells to undergo normal mitosis and G<sub>2</sub>/M to G<sub>1</sub> progression.

*Ada3* has been shown to be a core component of a number of HAT complexes. The authors used depletion of *Ada3* as an indication of disruption of only ATAC complex; however, deletion of *Ada3* would affect several HAT complexes and not just ATAC complex. Thus, deletion of *Ada3* would cause disruption of several HAT complexes that function in different phases of the cell cycle leading to defects in various phases of the cell cycle. Based on these findings, we propose the following working model of *Ada3* regulation of cell cycle progression. As part of a chromatin-remodeling complex, likely the STAGA complex, *Ada3* is recruited to and modifies the *c-myc* transcriptional regulatory elements to enhance *Skp2* transcription. This leads to destabilization of p27 by the SCF(*Skp2*) E3 ligase, resulting in increased Cdk2 activity and Rb phosphorylation to promote G<sub>1</sub>/S progression. Additionally, *Ada3*, by regulating the number of genes involved in mitosis, regulates cell division. Lastly, *Ada3* as part of ATAC and STAGA complex regulates transcription of various genes by recruiting HATs and acetylating histones. Combination of these functions led to severe cell cycle defect and embryonic lethality upon *Ada3* deletion (Fig. 9). Finally, although our studies here have focused on the role of *Ada3* in cell cycle progression, future studies using cell type- or stage-specific condi-

tional deletion of *Ada3* in mouse to assess its role in functions other than transcriptional activation, including optimal transcription elongation, mRNA export, and nucleotide excision repair, need to be explored (8, 46, 47).

In conclusion, we demonstrate that the evolutionarily conserved Ada3 protein as an essential component of HAT complex plays an important role in embryogenesis and cell division. Thus, our studies identify Ada3 as a novel component of the physiological regulation of mammalian cell cycle progression and set the stage for future studies to assess the role of Ada3 in cell cycle progression during *in vivo* physiological and pathological settings. Use of *Ada3<sup>FL/FL</sup>* mice should facilitate these analyses to functionally dissect the *in vivo* roles of Ada3.

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