Mammalian alteration/deficiency in activation 3 (Ada3) is essential for embryonic development and cell cycle progression

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Running Title: Ada3 regulates cell cycle progression

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

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

Conclusion: Ada3 is 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.

SUMMARY

Ada3 protein is an essential component of histone acetyl transferase containing coactivator complexes conserved from yeast to humans. We show here that germline deletion of Ada3 in mouse is embryonic lethal, and adenovirus-Cre mediated conditional deletion of Ada3 in Ada3FL/FL MEFs leads to several defects including i) severe proliferation defects which was rescued by ectopic expression of human Ada3 ii) delay in G1 to S phase of cell cycle due to accumulation of Cdk inhibitor p27 which was an indirect effect of c-myc gene transcription control by Ada3. We showed that this defect could be partially reverted by knocking down p27 iii) drastic changes in global histone acetylation and changes in global gene expression as observed in microarray analyses and iv) formation of abnormal nuclei, mitotic defects and delay in G2/M to G1 transition. 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 chromosomes 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 posttranslational modification of core histones (H2A, H2B, H3 and H4) is an essential process for altering chromatin structure (3,4). Histone acetyl transferases (HATs) and histone deacetylases (HDACs) are required to maintain steady state levels of acetylation (5). Several HAT enzymes, such as Gcn5 (General Control Nonderepressible 5), PCAF (p300/CBP associated factor), p300, CBP (CREB-binding protein) 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) and Spt3/Taf9/Gcn5 acetyltransferase complex (STAGA) (human homologs 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 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 HPV 16 E6-binding protein (15). Human Ada3 is the 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^{Flox/Flox} (Ada3^{FL/FL}) MEFs showed that Ada3 is required for efficient cell cycle progression through G1 to S transition as well as for proper mitosis. Detailed analyses in this system revealed an Ada3-c-myc-Skp2-p27 axis that controls G1 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 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 and isolation of mouse embryos and PCR genotyping.** Details concerning generation of conditional Ada3 knockout construct and Ada3 knockout mouse as well as PCR genotyping strategies, are described in Supplementary information.

Cell culture procedures and viral infections-Embryonic day 13.5 embryos were dissected from Ada3^{FL/+} 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 EGFP alone were purchased from University of Iowa (Gene transfer vector core). Adenovirus dose of 50 to 100 MOI diluted in 4ml serum free medium was added to cells in 100 mm culture dishes (at about 30% confluence) and incubated for 1 hour each at room temperature and at 37°C followed by 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-hAda3 vector, full length flag-hAda3 (15) was cloned into pMSCVpuro vector (Clontech). Retroviruses were generated by transiently transfecting this retroviral construct into Phoenix ectropic packaging cell line using the calcium phosphate co-precipitation method. The retroviruses were transduced into Ada3^{FL/FL} MEFs by 3 infections at 12h intervals using supernatant from transfected Phoenix cells to generate Ada3^{FL/FL} MEFs expressing flag-hAda3. Scrambled shRNA (5'GGTTAAACCTTACGATG3') or p27 shRNA (5'GTGGAATTTCGACTTTGACAG3') was introduced into Ada3^{FL/FL} MEFs by using 3 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 48h until complete loss of uninfected cells.

**Proliferation assay, colony formation efficiency assay, and cell cycle analysis.** To perform proliferation assays, one day after adenovirus infection, cells were plated at different numbers in 6 well plates in triplicates [5 x 10^4 (for counting on day 3), 2.5 x 10^4 (for counting on day 5), 1.25 x 10^4 (for counting on day 7) and 0.625 x 10^4 (for counting on day 9)] and counted at the indicated time points. For colony formation assay, cells 3 days post adenovirus-infection were trypsinized and plated 1000 cells per 100mm 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, two days after plating and adenoviral infection of 2 X 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 G2/M phase, 48 hours 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 Supplementary information.

**In vitro Kinase Assay**-In vitro kinase assay was performed using purified Histone H1 (Roche) 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$_2$VO$_4$, 1 mM NaF; and protease inhibitor mixture), and Cdk complex was recovered by immunoprecipitation with either 2 μg of 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$_2$, 1 mM dithiothreitol, 0.1 mM Na$_2$VO$_4$, 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 (500ng) in kinase buffer containing 10 mM β-glycerophosphate, 33 μM ATP, and 10 μCi of [γ-$^3$P]ATP (10 mCi/ml, 6000 Ci/mmoll) at room temperature for 20 min. The products were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes (PVDF), and autoradiographed.

**Analysis of the p27 Protein Turnover-Ada3FL/FL** MEFs were plated in 100-mm dishes and infected with control or Cre adenoviruses. For analyzing p27 protein half life in exponentially growing cells, two days after adenovirus infection, cells were treated with 50 μg/ml of cycloheximide (Sigma) and harvested at indicated time points. For analyzing p27 protein half life in serum starved cells, two days after adenovirus infection, cells were starved for 72 h in 0.1% serum containing medium. Subsequently, 50 μg/ml of cycloheximide was added to the medium and cells harvested at 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 μg of total RNA was used for reverse transcriptase reaction using SuperScript™ II reverse transcriptase (Invitrogen). Real-time PCR quantification was performed in triplicate using SYBR Green PCR master mix (Applied Biosystems) and the primers listed in Supplementary Table S3. Expression levels were normalized against β-actin RNA levels, and the results were calculated by the ΔΔC$_{\text{t}}$ method.

**Chromatin immunoprecipitation experiments**-Approximately 0.7 million Ada3FL/FL 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 hours and then stimulated with complete medium (DMEM + 10% FCS) for 0-60 minutes as indicated for each experiments in Figure 8C. ChIP experiment was performed by using ChIP-IT Express kit from Active Motif. PCR amplification was performed using primers for the c-myc enhancer (forward, 5'-CTAGAACCAATGACAGAGC-3'; reverse, 5'-CTCCCCAGGACAAAACCAAGC-3') and for Skp2 promoter (forward, 5'-GCCATCGAGACCCCGGAGAT-3'; reverse, 5'-TGATGCCCTTCCAGACGCTGT-3'). Control PCR was performed using primers for the c-myc distal site (forward, 5'-ACACACCTTGATCCCGTGTT-3'; reverse, 5'-CCCAAGTCAATGAGAAG-3') and for Skp2 distal site (forward, 5'-GATGGTGGCGGTACCTTTGTTTG-3'; reverse, 5'-GATAAGGATGCACTCTGGGGC-3'). PCR products were analyzed on 2% agarose/TEB gels with ethidiumbromide 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® Expression System**-Ada3 baculoviral construct information and recombinant protein purification is detailed in Supplementary information.

**Microarray analyses**-Protocol for microarray analyses is described in Supplementary information. The microarray data from this publication has been submitted to the GEO database and has been assigned the following Series record: GSE37542.
RESULTS

Deletion of Ada3 leads to early embryonic lethality in mice-The targeting construct generated using the recombineering technique (Supplementary Figure S1A; see methods section) was electroporated into an ES cell line derived from the 129/Ola strain of mice. Screening of resultant neomycin-resistant colonies yielded 3 correctly targeted clones (Supplementary Figure 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 are viable and fertile, and exhibit no gross abnormalities compared to 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 EIIa promoter-driven Cre (B6.FVB-Tg (EIIa-Cre) C5379Lmgd/J). EIIa 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 X C57BL/6 background were viable and fertile, and their median life span of more than 18 months was comparable to that of their control littersmates. 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 haplo-insufficiency 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 (Supplementary Figures S1C and S1D). Since no homozygous mutant embryos were recovered beyond embryonic day 8.5 (E8.5; Table 1), blastocysts were isolated at 3.5 days post-coitum (dpc) and genotyped directly by PCR (Supplementary Figure 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 (Supplementary Figure S1F). PCR analysis revealed that approximately 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 since 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 if 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 methods section). As seen in Supplementary Figure 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 adenoviral infection, and cells were counted at the indicated time points up to 9 days. Notably, Ada3-deleted MEFs exhibited significantly slower rate of proliferation as compared to control MEFs (Figure 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 retroviralrly introducing human Ada3 (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 (Figure 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 (Figure 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, while 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 (Figure 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 (Figures 1C and 1D).

Ada3 is required for cell cycle progression through G1 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 if Ada3 plays a role in cell cycle progression, Ada3FL/FL MEFs were infected with control and Cre adenoviruses, arrested in G0/G1 by serum-deprivation for 72 hrs and then synchronously released into cell cycle by serum stimulation. FACS-based cell cycle analysis of propidium iodide-stained cells showed significant delay in G1 to S progression in Ada3-deleted MEFs as compared to control MEFs (Figure 2A). Of note, the relative distribution of S phase in Ada3-null MEFs after 20 h of serum stimulation was about half (31.6 ± 2.33 S.E. %) of the control virus infected MEFs (56.05 ± 4.71 S.E. %) (Figure 2B). These results demonstrate that conditional deletion of Ada3 leads to delay in G1 to S progression in MEFs, indicating an essential role of Ada3 in efficient G1/S progression.

Elevated p27Kip1 levels and impaired Rb phosphorylation upon conditional Ada3 deletion—Given the delay in G1/S progression imposed by induced Ada3 deficiency, we examined the status of key proteins known to control the G1/S transition. A well-established and critical event during G1 to S progression is the phosphorylation of Rb by Cyclins (particularly D, E and A)-dependent kinase (Cdk) complexes, such as Cdk4/6 and Cdk2 (24,25); phosphorylation of Rb leads to its release from Rb/E2F complexes, relieve 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 G1 to S phase (26,27). Therefore, we carried out western blotting of cell lysates obtained from control vs. conditional Ada3-deleted MEFs released into synchronous cell cycle progression to assess the levels of proteins relevant to G1 to the S phase transition. Notably, while 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 compared to control cells (Figure 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. Whereas the Cdk4/6 kinase activity was comparable between control and adeno-Cre infected MEFs (Figure 3B), the level of Cdk2 kinase activity was substantially reduced in Cre infected MEFs as compared with control MEFs (Figure 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 transcriptional or post-transcriptional level. Real-time PCR analysis showed that serum stimulation resulted in a marked reduction in the levels of p27 mRNA both in the control and Cre infected cells (Figure 4A); furthermore, the levels of p27 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 is a prominent mechanism to control Cdk inhibitor levels (28), we compared the half-life of p27 protein in WT vs. Ada3-deleted MEFs using two distinct experimental formats: the first one utilized exponentially-growing cultures while the second one utilized cells first arrested in G1 by serum-deprivation for 72h followed by synchronous release into cell cycle by serum addition. In each case, Ada3FL/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 at about 2 h and 40 min in exponentially growing MEFs whereas in growth-arrested cells at about 3 h and 30 min (Figures 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 vs. 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 G1/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 if this is the case, we generated stable p27 knockdown Ada3<sup>FL/FL</sup> MEFs (Ada3<sup>FL/FL/p27shRNA</sup>) by infecting Ada3<sup>FL/FL</sup> MEFs with a retrovirus expressing a p27-specific shRNA followed by selection in puromycin, which resulted in a significant knockdown of p27 expression in these cells (Figure 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 (Figure 5B). Notably, a partial but clear rescue of the G1/S delay was observed in p27 shRNA-expressing cells, as seen by a much larger percentage of cells entering the S phase (41.4 ± 3.5 S.E. % in p27 shRNA expressing conditionally deleted Ada3 MEFs vs. 31.6 ± 2.33 S.E. % in Ada3 deleted MEFs at 20 h; compare Figure 5C with Figure 2B). Importantly, the levels of Cyclin A, which is known to be expressed during G1/S transition and to peak in the S phase, as well as hyperphosphorylation of Rb, were essentially fully rescued by p27 shRNA knockdown (Figure 5D; compare with Figure 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 G1/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 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 vs. control MEFs (Figures 6A and 6B). 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 if Ada3 deletion alters c-myc mRNA levels and if Ada3 directly binds to c-myc promoter. Indeed, analysis of control vs. 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 (Figures 6C and 6D). Consistent with this, we observed lower occupancy of mouse Skp2 promoter by c-myc upon deletion of Ada3, which supports our results (Supplementary Figure S3). Finally, to establish that Ada3 indeed participates in the enhancement of myc gene transcription, we carried out ChIP analysis to assess if 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 -5kb) was seen upon serum stimulation of MEFs (Figure 6E). As expected, we did not detect any signals after IP with anti-Ada3 antibody in cells infected with aden-Cre. These results therefore support the existence of a novel cell cycle-associated, Ada3-regulated signaling pathway that promotes G1/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 G1/S transition in Ada3 deleted MEFs after knock down 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, we examined if 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 compared to control MEFs (Figure 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 G1 phase and subsequent release. There was a dramatic down-regulation of H3-K9 acetylation and a slight decrease in acetylation of H2B-K5 and H3-K14 lysine residues in Ada3-deleted MEFs compared to control-MEFs, whereas this defect was rescued in Ada3FL/FL MEFs reconstituted with exogenous human flag-Ada3 (Figure 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 G1 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 delayed transition through S phase.

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 for immunoblotting with two important HATs such as p300 and PCAF. Indeed, deletion of Ada3 caused drastic downregulation of p300 and PCAF in MEFs (Figure 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 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 if 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 if Ada3 directly modulates the activity of known HAT enzyme 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 tranferase enzyme on histone substrates was measured. As seen in Figure 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 (Figure 7E). Thus, Ada3 manifests its effect on histone acetylation by maintaining 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 downregulated and 928 genes were upregulated ≥ 1.5 fold upon Ada3 deletion (Supplementary Table S1). Validation of some of the deregulated genes from microarray by real time PCR showed good co-relation with the microarray data (Supplementary Figure S4). Ingenuity pathway analyses showed most of the genes affected were involved in controlling cell growth, proliferation and cell death (Supplementary Table S2, top biological functions affected – cell growth and proliferation (386 genes) and cell death (359 genes). The top network affected was RNA post-transcriptional modification and cellular assembly and organization, whereas cell cycle, endocrine system development & function and cancer was the 3rd most affected network (Supplementary Figure S5). Notably, c-myc and Skp2 genes that we described above were downregulated 1.4 fold 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 was performed on asynchronous populations, whereas the real time PCR data was performed on synchronous cells (Figures 6A and 6C). 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 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 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 as compared to control MEFs. Ada3-deficient MEFs showed various nuclear abnormalities such as fragmentation, lobulation and multinucleation (Figure 8A). Compared to 13.08 ± 2.39 S.E. % control MEFs, 83.41 ± 3.45 S.E. % of Ada3-deficient MEFs showed abnormal nuclei (Figure 8B). Live imaging of cells for 24 hours showed 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 afterwards and were unable to divide. In other cases, cell division resulted in the formation of anucleated daughter cells (Representative images shown in Supplementary Figure 7). 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 G2/M to G1 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 G2/M to G1 transition. To examine this effect, we synchronized control and Cre-adenovirus infected Ada3<sup>FL/FL</sup> MEFs at G2/M checkpoint by treating them with Nocodazole, released from synchrony and followed by cell cycle analysis using flow cytometry (Figure 8C). Nocodazole synchronized Ada3-deleted MEFs showed lower percentage of cells in G2/M phase (61%) at 0 h time point as compared to control MEFs (80%) (Figure 5C). On the contrary, we observed higher percentage (20%) of Ada3-deleted MEFs in G1 phase compared to control MEFs (7%) after synchronization. We speculate that Ada3-deficient MEFs that are exhibiting a delay in G1 to S transition were unable to get completely synchronized at G2/M checkpoint as these cells are potentially moving slowly through the G1 to S transition and require a prolonged treatment with Nocodazole in order to show a complete synchronization as seen in control MEFs. When we compared the percentage of cells moving into G1 phase on release from Nocodazole treatment in both Ada3-deficient and control MEFs, a significant impairment in G2/M to G1 transition in Ada3-deleted MEFs was observed (Figure 8D). Taken together, these results demonstrate a critical role of Ada3 in both G1 to S as well as G2/M to G1 transition in MEFs, indicating that the cell proliferation defect observed in Ada3-deficient MEFs is due to a combined defect in G1 to S as well as G2/M to G1 transition.

**DISCUSSION**

Regulated cell cycle entry and progression are essential for precise developmental programs as well as to maintain organ homeostasis in adult animals. While 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 an HPV E6 oncoprotein partner as well as a coactivator of cell cycle checkpoint regulator and tumor suppressor p53 (15,20). Several in vitro reports have shown that Ada3 is an essentially universal component of a multitude of HAT-based transcriptional regulatory complexes, 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 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 such as Drosophila where Ada3 deficiency is associated with arrest in early development (36). However, this report 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, Trap, p300, 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 Trap 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 days), p300 (E9.5-E10.5 days) and Atac2 (E11.5 days) in comparison to E3.5 block observed in Ada3-null mice. The relatively early developmental arrest of Ada3-null mice compared to 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 is somewhat surprising and
sustained defects exhibit to control of c-myc gene transcription by Ada3. This result is consistent with independent findings from two groups of Ada3 to c-myc enhancer elements. This result is consistent with our observations, it is noteworthy that c-myc knockout 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 if this is the case.

Although, regulation of p27 protein stability by Ada3 through control of c-myc transcription forms an important basis for G1/S transition defects, we were not able to fully rescue the defect in cell cycle by using p27 shRNA, suggesting involvement of other cellular pathways. To this end, examining global histone acetylations in Ada3-deficient cells revealed dramatic defects in histone acetylation. Since Ada3 forms a core structural component of various different HAT complexes in the cell, 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 RNA post-transcriptional modification and cellular assembly and organization network as the top affected network, with cell cycle, endocrine system development & function and cancer network as the 3rd 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 (Supplementary table S2), which could be linked to the early embryonic lethality observed upon knockout 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 G2/M to G1 phase progression upon deletion of Ada3. Our observed phenomenon of cell division defects upon deletion of Ada3 is consistent with a recently published report (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 was attributed to stabilization of Cyclin A upon disruption of ATAC complex. While we did not observe an increase in Cyclin A levels (in fact the converse) in our system, we did observe 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, 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 cell cycle leading to defects in various phases of 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 G1/S progression. Additionally, Ada3, by regulating 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 (Figure 9).

Finally, while our studies here have focused on the role of Ada3 in cell cycle progression, future studies using cell type or stage-specific conditional 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, needs 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 Ada3FL/FL mice should facilitate these analyses to functionally dissect the in vivo roles of Ada3.
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FOOTNOTES

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1 These authors contributed equally to this work
2 Supported by Susan G. Komen Postdoctoral fellowship (KG111248)
3 The abbreviations used are: HPV, human papillomavirus; Ada3, alteration/deficiency in activation 3; HAT, histone acetyltransferase; MEF, mouse embryonic fibroblast; CDK, cyclin-dependent kinase; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation.

FIGURE LEGENDS

FIGURE 1. Ablation of Ada3 causes proliferation defect in MEFs. (A) Growth curves of Ada3^{FL/FL} (left) and Ada3^{FL/FL/fhAda3} (right) MEFs after Control (Ctrl) adenovirus or Cre (Cre) adenovirus infection. Data are means ± 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) protein, whereas only hAda3 is seen in Cre adenovirus-infected cells. (C) Colony formation assay; crystal violet staining of indicated cells infected with control virus or Cre adenovirus grown for 10 days. (D) Western blotting of lysates from C showing exogenous and endogenous Ada3.

FIGURE 2. Ada3 disruption delays G1 to S transition in MEFs. (A) Control (Ctrl) or Cre infected Ada3^{FL/FL} MEFs were serum starved for 72 hours and then released from synchrony as described in materials and methods and processed for PI staining followed by FACS analysis. Cells in different phases of 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 ± S.E. from three independent experiments (***p = 0.0096, two-tailed Student’s t-test)

FIGURE 3. Effect of Ada3 depletion on expression of cell cycle regulator proteins and Cdk2 kinase activity. (A) Ada3^{FL/FL} MEFs infected with Control (Ctrl) and Cre adenoviruses serum starved for 72 hours, released from synchrony as described in materials and methods and processed for immunoblot analysis of indicated cell cycle proteins. (B) Anti-Cdk2 or anti-Cdk4/6 immunoprecipitates performed using 300 μg extracts of Ada3^{FL/FL} 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.

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 figure 2. Signals were normalized to β-actin levels and plotted relative to the level of p27 mRNA in starved control (Ctrl) cells. Error bars show mean ± SE from three independent experiments. (B-E) Ada3 deletion in MEFs extends p27 half life. (B) 48 hours after adenovirus infection, MEFs were treated with 50 μg/ml of cycloheximide, harvested at indicated time points and p27 and β-actin protein levels were analyzed by immunoblotting. (C) The intensity of p27 bands were quantified by densitometry, normalized to β-actin using ImageJ software and plotted against time of cycloheximide treatment. Each decrease of 1 unit of log 2 is equivalent to one half-life. The lines were generated by linear regression formula. (D) After 48 hours of adenovirus infection, MEFs were starved using 0.1% serum containing medium for 72 hours and subsequently
treated with 50 μg/ml of cycloheximide and harvested at the indicated time points. Cell lysates were analyzed by western blotting using antibodies against p27 and β-actin. (E) Graph made from experiment in (D) by using the same procedure as in (C).

**FIGURE 5.** p27 depletion partially rescues G1 to S transition defects seen in Ada3 null MEFs. (A) *Ada3FL/FL* 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 *Ada3FL/FL* MEFs expressing p27 shRNA that were infected with either Control (Ctrl) or Cre adenoviruses and synchronized as in Figure 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 re-stimulation. Error bars indicate mean ± S.E. from three independent experiments (D) Immunoblotting of protein samples from B showing rescue of hyperphosphorylated Rb and Cyclin A levels.

**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 figure 2. Signals were normalized to β-actin levels and plotted relative to the level of Skp2 mRNA in starved control cells. Error bars represent mean ± SE 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 figure 5. Signals were normalized to β-actin levels and plotted as in A. Error bars show mean ± SE 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 3h, 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 Ada3FL/FL* MEFs 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 hours (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.

**FIGURE 7.** Ada3 deletion abrogates histone acetylation by destabilizing various HATs. Western blotting analysis of lysates from asynchronous (A and C) or serum re-stimulated (B) *Ada3FL/FL* or *Ada3FL/FL*Ada3* MEFs infected with Control (Ctrl) or Cre adenoviruses using indicated antibodies (D-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.

**FIGURE 8.** Abnormal cell division and delayed G2/M to G1 transition in Ada3 deleted cells. (A) Images of *Ada3FL/FL* 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 Geimsa stain and scored for abnormal nuclei (at least 100 cells from each group were counted). Error bars show mean ± S.E. from three independent experiments (C) Control and Cre adenovirus infected MEFs were treated with 20 h with nocodazole and were harvested at indicated time points after release, stained with PI and subjected to FACS analysis. (D) Graph showing percentage of cells entering G1 phase after release from nocodazole treatment at various time points from experiments as in C. Error bars are mean ± S.E. from three independent experiments (*p = 0.034; ** p = 0.0038 and 0.007 for 4 h and 8 h, respectively, by two tailed Student’s t-test).

**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 G1-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 cells progress from G1 to S phase of cell cycle. Additionally, Ada3 through controlling global histone acetylation, controls transcription of various genes involved in cell division and is required for cells to undergo normal mitosis and G2/M to G1 progression.
Table 1. Genotype analysis of embryos from heterozygous intercrosses

| Stage | Total 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        |

Table 2. List of deregulated genes involved in cell division and DNA replication

| Gene Symbol | Gene Title | Fold Down-regulated |
|-------------|------------|---------------------|
| Kifc1 /// LOC100044 | kinesin family member C1 /// similar to Kifc1 protein | 2.0 |
| Nfkbi1 | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1 | 2.0 |
| Fbxo5 | F-box protein 5 | 1.8 |
| Cenpf | centromere protein F | 1.8 |
| Cdc6 | cell division cycle 6 homolog (S. cerevisiae) | 1.7 |
| Kntc1 | kinetochore associated 1 | 1.7 |
| Baz1b | bromodomain adjacent to zinc finger domain, 1B | 1.6 |
| Mlf1ip | myeloid leukemia factor 1 interacting protein | 1.6 |
| Myh10 | myosin, heavy polypeptide 10, non-muscle | 1.6 |
| Kif11 | kinesin family member 11 | 1.6 |
| Cen2 | cyclin A2 | 1.6 |
| Smc2 | structural maintenance of chromosomes 2 | 1.6 |
| Plk1 | polo-like kinase 1 (Drosophila) | 1.5 |
| Bub1b | budding uninhibited by benzimidazoles 1 homolog, beta (S. cerevisiae) | 1.5 |
| Aspm | asp (abnormal spindle)-like, microcephaly associated (Drosophila) | 1.5 |
| Anln | anillin, actin binding protein | 1.5 |
| Zwilch | Zwilch, kinetochore associated, homolog (Drosophila) | 1.5 |
| Mki67 | antigen identified by monoclonal antibody Ki 67 | 1.5 |
| Mad2l1 | MAD2 mitotic arrest deficient-like 1 (yeast) | 1.5 |
| Smc4 | structural maintenance of chromosomes 4 | 1.5 |
| Cdc8 | cell division cycle associated 8 | 1.5 |
| Kif20b | kinesin family member 20B | 1.5 |
| Hells | helicase, lymphoid specific | 1.5 |
| Cenb1 | cyclin B1 | 1.5 |
| Cdc3 | cell division cycle associated 3 | 1.5 |
Ada3 regulates cell cycle progression

| Gene   | Description                                                                 | Fold Change |
|--------|-----------------------------------------------------------------------------|-------------|
| Nuf2   | NUF2, NDC80 kinetochore complex component, homolog (S. cerevisiae)           | 1.5         |
| Ndc80  | NDC80 homolog, kinetochore complex component (S. cerevisiae)                 | 1.5         |
| Birc5  | baculoviral IAP repeat-containing 5                                          | 1.5         |
| Bub1   | budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)              | 1.5         |
| Suv39h2| suppressor of variegation 3-9 homolog 2 (Drosophila)                        | 1.5         |
| Aurkb  | aurora kinase B                                                             | 1.5         |
| Weel   | WEE 1 homolog 1 (S. pombe)                                                  | 1.5         |

| Gene   | Description                                                                 | Fold Change |
|--------|-----------------------------------------------------------------------------|-------------|
| Kitl   | kit ligand                                                                  | 1.9         |
| Prim1  | DNA primase, p49 subunit                                                    | 1.7         |
| Mcm7   | minichromosome maintenance deficient 7 (S. cerevisiae)                      | 1.7         |
| Ccne2  | cyclin E2                                                                   | 1.7         |
| Polal  | polymerase (DNA directed), alpha 1                                          | 1.7         |
| Dtl    | denticleless homolog (Drosophila)                                           | 1.7         |
| Ccne6  | cell division cycle 6 homolog (S. cerevisiae)                               | 1.7         |
| Chtf18 | CTF18, chromosome transmission fidelity factor 18 homolog (S. cerevisiae)   | 1.7         |
| Nfib   | nuclear factor I/B                                                           | 1.6         |
| Prim1  | DNA primase, p49 subunit                                                    | 1.6         |
| Orc1l  | origin recognition complex, subunit 1-like (S.cereviaiae)                   | 1.6         |
| Rrm1   | ribonucleotide reductase M1                                                  | 1.6         |
| Rpal   | replication protein A1                                                      | 1.6         |
| Cdt1   | chromatin licensing and DNA replication factor 1                             | 1.6         |
| Gins2  | GINS complex subunit 2 (Psf2 homolog)                                       | 1.5         |
| Rbhp4  | retinoblastoma binding protein 4                                             | 1.5         |
| Chaf1b | chromatin assembly factor 1, subunit B (p60)                                | 1.5         |
| Tk1    | thymidine kinase 1                                                           | 1.5         |

‡Genes downregulated at least 1.5 fold upon loss of Ada3 as obtained from microarray analyses. The genes were classified based upon gene ontology biological processes.
Figure 1

**A**

*Ada3^{FL/FL}*

![Graph showing cell number over days with Ctrl and Cre conditions.](image)

**B**

*Ada3^{FL/FL}/fhAda3*

![Graph showing cell number over days with Ctrl and Cre conditions.](image)

**C**

*Ada3^{FL/FL}*

![Western blot images for Ada3 and Hsc70 with Ctrl and Cre conditions.](image)

**D**

*Vector, Flag-hAda3*

![Western blot images for mAda3 and β-actin with Ctrl and Cre conditions.](image)
**Figure 2**

A

| Hours after serum re-stimulation |
|---------------------------------|
| Ctrl                            |
| Cre                             |

B

% cells in S phase vs. Hours after serum re-stimulation

- **Ctrl**
- Cre

**Ada3**

---

*Downloaded from [URL]*

*By guest on March 24, 2020*
Figure 3

A

|        | Ctrl | Cre |
|--------|------|-----|
| 0      | 16   | 20  |
| 16     | 20   | 24  |
| 24     | 28   | 0   |
| 28     |      |     |

Serum re-stimulation (h)

- Ada3
- Cdk2
- Cdk4
- Cdk6
- p16
- p21
- p27
- Cyclin A
- Cyclin E
- Cyclin D
- Rb
- Hsc70

B

|        | Ctrl | Cre |
|--------|------|-----|
| 0      | 0    | 20  |
| 0      |      | 20  |
| 20     |      |     |

Serum re-stimulation (h)

- IP:CDK2
- IP:CDK2
- IP:CDK4/6
- WB:CDK2
- WB:CDK4
- WB:p27
- WB:CDK2
- WB:CDK4
- Rb
- Histone H1
Figure 4

A: Relative p27 mRNA levels

B: Cycloheximide chase (h) for p27 and β-actin

C: Log2(band intensity) over time for Ctrl and Cre

D: Cycloheximide chase (h) for p27 and β-actin

E: Log2(band intensity) over time for Ctrl and Cre
Figure 5

B

Hours after serum re-stimulation

0   16   20   24   28

Ctrl

Cre

C

Ada3

% cells in S phase

Ctrl

Cre

D

Serum re-stimulation (h)

0   16   20   24   28   0   16   20   24   28

Rb

Cyclin A

Ada3

β-actin

A

Scrambled shRNA

p27 shRNA

β-actin
Figure 6

A

Relative Skp2 mRNA levels

0 16 20 24 28
 hours

B

Ctrl Cre

0 16 20 24 28
Serum (h)

Skp2

Ada3

Hsc70

C

Relative c-myc mRNA levels

0 1 3 5 7
 hours

D

Ctrl Cre

0 1 3 5
Serum (h)

c-myc

Ada3

β-actin

E

Ctrl Cre

Asyn. 0 30 60 Asyn. 0 30 60
Serum (min)

Input Ada3

RNA Pol II Mouse IgG

Input Ada3

RNA Pol II Mouse IgG
Figure 7

| Protein | Ctrl | Cre |
|---------|------|-----|
| H2A-K5  |      |     |
| H2A     |      |     |
| H2B-K5  |      |     |
| H2B     |      |     |
| H3-K9   |      |     |
| H3-K56  |      |     |
| H3      |      |     |
| H4-K8   |      |     |
| H4      |      |     |
| Ada3    |      |     |
| β actin |      |     |

| Protein | Ctrl | Cre |
|---------|------|-----|
| Ada3FL/FL |   0  | 16  | 20  | 24  | 28  | 0   | 16  | 20  | 24  | 28  |
| Serum (h) |     |     |     |     |     |
| H2B-K5   |     |     |     |     |     |
| H2B      |     |     |     |     |     |
| H3-K9    |     |     |     |     |     |
| H3       |     |     |     |     |     |

| Protein | Ctrl | Cre |
|---------|------|-----|
| Ada3FL/FL/hfAda3 |   0  | 16  | 20  | 24  | 28  | 0   | 16  | 20  | 24  | 28  |
| Serum (h) |     |     |     |     |     |
| H2B-K5   |     |     |     |     |     |
| H2B      |     |     |     |     |     |
| H3-K9    |     |     |     |     |     |
| H3       |     |     |     |     |     |

| Protein | Ctrl | Cre |
|---------|------|-----|
| p300    |      |     |
| PCAF    |      |     |
| α-tubulin |      |     |
| β-actin |      |     |
Figure 7 (Continued)

| Core histones | + | + | + | + | + | + | + | + |
|---------------|---|---|---|---|---|---|---|---|
| \(^3\text{H} \text{ Acetyl Co-A} | + | + | + | + | + | + | + | + |
| \(\text{p300 (10ng)} | - | - | + | + | + | + | + | + |
| \(\text{Ada3 (ng)} | - | + | - |   |   |   |   |   |

HAT assay

[Image of HAT assay with bands for Ada3, H1, H3, H2A/H2B, H4]

Ponceau

[Image of Ponceau stain with bands for Ada3, H3, H2A/H2B]

| Histone H3 | + | + | + | + | + | + | + | + |
|------------|---|---|---|---|---|---|---|---|
| \(^3\text{H} \text{ Acetyl Co-A} | + | + | + | + | + | + | + | + |
| \(\text{p300 (10ng)} | - | - | + | + | + | + | + | + |
| \(\text{Ada3 (ng)} | - | + | - |   |   |   |   |   |

HAT assay

[Image of HAT assay with bands for Ada3, H3, H2A/H2B]

Ponceau

[Image of Ponceau stain with bands for Ada3, H3, H2A/H2B]
Figure 8

A

Multi lobed nucleus
Bi lobed nucleus
Multi nuclei
Fragmented nucleus
Tri lobed nucleus
Multi nuclei

B

Cells with abnormal nuclei
p < 0.001

C

Hours after release from Nocodazole treatment

D

Ada3^{FL/FL}

% cells entering G1 phase

% Cells

Ctrl
Cre

% cells entering G1 phase

Ctrl
Cre

Hours

* *
Ada3 – a core structural component of multiple HAT complexes

Regulates stability of HATs and global histone acetylation

Binds to myc enhancer and regulates c-myc transcription

c-myc in turn binds to Skp2 promoter and regulates Skp2 transcription

Skp2 as an E3 ubiquitin ligase regulates p27 protein levels

p27 degradation leads to increased Cdk2 kinase activity and hyperphosphorylation of Rb

Normal G1 to S transition

Controls transcription of several genes involved in mitosis

G2/M to G1 transition

Figure 9
Mammalian alteration/deficiency in activation 3 (Ada3) is essential for embryonic development and cell cycle progression
Shakur Mohibi, Channabasavaiah Basavaraju Gurumurthy, Alo Nag, Jun Wang, Sameer Mirza, Yousaf Mian, Meghan Quinn, Bryan Katafiasz, James Eudy, Sanjit Pandey, Chittibabu Guda, Mayumi Naramura, Hamid Band and Vimla Band

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