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Award Number:
W81XWH-11-1-0687

Title
Modulation of TIP60 by Human Papilloma Virus in Breast Cancer

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REPORT DATE: April 2013

TYPE OF REPORT
Annual Summary

PREPARED FOR: U.S. Army Medical Research and Material Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
April 2013	Annual Summary	15 August 2011---24 January 2013

4. TITLE AND SUBTITLE

Modulation of TIP60 by Human Papilloma Virus in Breast Cancer

5a. CONTRACT NUMBER
W81XWH-11-1-0687

5b. GRANT NUMBER
W81XWH-11-1-0687

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Ashish Gupta

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Virginia

Charlottesville, VA-22904

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We examined the role of HPVE6 on the modulation of Tip60 in Breast epithelial cells. HPVE6 oncoprotein can destabilize the Histone acetyl transferase TIP60 in breast epithelial cells. Using the inhibitors of proteasomal pathway we found that the destabilization of Tip60 by E6 is through its protein degradation. Our Ubiquitination assays result suggested that this degradation of TIP60 might not involve the polyubiquitination dependent post translational modification. We hypothesize that E6 might facilitate the interaction of TIP60 with its E3 ligase and thus leads to its depletion. We aim to find the E3 ligase involved in E6 mediated TIP60 degradation. We intend to examine the role of TIP60 degradation by HPV virus and its significance in malignant transformation of the breast epithelial cells.

15. SUBJECT TERMS
Breast Cancer, HPV, TIP60, E6, HAT

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
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Introduction:

HPV and Tip60 modulation in Breast Cancer

Besides the various genetic and environmental factors thought to cause breast cancer, viruses have also been implicated. At least six human viruses are linked with cancers, namely Epstein- Barr virus (EBV), Hepatitis B Virus (HBV), Hepatitis C virus (HCV), Human Papilloma virus (HPV), Human T-cell lymphotropic virus (HTLV-1) and Kaposi's associated Sarcoma virus (KSHV) (1). Human Papilloma Virus (HPV) is known to cause cervical cancer and head and neck cancer and has been proposed to play a role in breast cancer. Several reports have shown the presence of high and low risk HPVs in breast tumors and cancer cells. For example, a paper from Dr. Harold zur Hausen (who received a recent Nobel Prize for his discovery of the oncogenic role of HPV) shows that >85% of breast cancers have evidence of high or low risk HPV infection (2) while another paper found 23-39% of breast cancers positive for high risk HPV (3). In addition, HPV immortalizes normal mammary epithelial cells (4).

Tat Interacting Protein 60 (Tip60/KAT5) is a lysine acetyl transferase involved in multiple cellular pathways like transcriptional regulation, steroid receptor function, DNA repair pathways (5, 6). It is involved in the activation and repression of promoters by acetylating histone tails proteins (7, 8). Besides this, Tip60 is known to acetylate p53, ATM kinase, Androgen receptor and other proteins and thus regulate them by changing their functional activity (9, 10). In this study we proposed to test whether E6 can degrade Tip60 in breast epithelial cells and the significance of such degradation for breast epithelial cell immortalization. Since tumor suppressors targeted by viral oncogenes are often inactivated in cancers by virus-independent mechanisms, we will also test whether the Tip60 tumor suppressor protein is inactivated in breast cancers by HPV-independent pathways.

Supplemental Aim: Determining the role of Adenovirus in destabilizing Tip60 and Breast Cancer

Like HPV Adenoviruses are also double stranded DNA viruses that infect human and rodent cell lines, occasionally transform them and cause tumors in animal models. The host cell challenges the virus in multifaceted ways to restrain viral gene expression and DNA replication, and sometimes even eliminates the infected cells by programmed cell death. To combat these challenges, adenoviruses abrogate the cellular DNA damage response pathway. Like HPV, Adenovirus also degrades or inactivates many cellular proteins like p53 and inactivates Rb (11-13). Although Adenovirus are not know to be

directly related to breast cancer, the recent hypothesis of “Hit and Run” and wide exposure of humans with common upper respiratory tract infection caused by adenovirus make us hypothesize that adenovirus can also be a etiological agent or can augment the breast epithelial cells transformation and cancer.

Body:

Testing HPVE6 can degrade Tip60 in breast epithelial cells

Task1: Generation of Tip60 antibody for Tip60 Western

Our first goal is to generate a monoclonal antibody against Tip60 that can be used to detect Tip60 in Western blotting analysis. For it we purified the His-Tip60 protein using Ni-NTA affinity purification and used it as antigen for polyclonal antibody generation in mice. After conforming the polyclonal antibody generation against Tip60 antigen in immunized mice sera, the spleen cells were collected from immunized mice and co-cultured with myeloma cells. These cells were cultured in selective HAT medium to select for fused cells called Hybridoma cells. These hybridoma cells were cultured and tested for monoclonal antibody generation against Tip60 by ELISA.

After confirming the synthesis of monoclonal antibody generation by hybridoma clones in ELISA, the culture medium were tested for Western blotting. U2OS cells were cultured in subconfluent condition and transfected with 30nM siRNA oligo targeting GL2 firefly Luciferase as a control or siTip60 oligo targeting Tip60 mRNA using Lipofectamine RNAimax transfection reagent. Cells were harvested 72 hrs after transfection and lysed for Western blotting. Western blotting result showed the presence of Tip60 in control siGL2 knockdown lane but not in Tip60 knockdown lane showing the specificity of our generated monoclonal antibody (Figure1A). Next we tested whether our in house generated monoclonal antibodies works in immunepreceipitation. 293T cells were fractionated into cytosolic and nuclear fractions. We did immunopreicipitation using Tip60 monoclonal antibodies or control IgG from both the fractions. Western blot results showed that Tip60 is immunoprecipitated only in Tip60 IP lanes in both cytosolic and nuclear fractions. IgG immunoprecipitated lanes does not show any Tip60 showing the specificity of generated Tip60 monoclonal antibodies (Figure1B).

Task2: Construction of E6 oncogene and Tip60 gene in mammalian expression vectors and effect of E6 oncoprotein on Tip60 degradation.

To investigate the effect of HPVE6 on Tip60, we first generated the clone of these genes in mammalian expression vectors. To generate the clone of Tip60 we amplified

the gene from cDNA using specific primers. This amplified product was cloned in pCDNA3 mammalian expression vector under *BamHI* and *XhoI* sites. Similarly we amplified E6 gene from cDNA using specific primers and cloned it in pCDNA3 mammalian expression vector under *EcoRI* and *XhoI* sites. After generating the mammalian expressing constructs for Tip60 and HPVE6, we try to investigate the effect of E6 on Tip60 in breast epithelial cells. Sub-confluent MCF10A cells were transfected with Myc-Tip60 alone or in combination with pCDNA3 or E6 clone. Cells were cultured for 48hrs post transfection before harvesting. Cells were lysed and equal amount of proteins were loaded for Western blotting by indicated antibodies (Figure2). The Western result showed that Tip60 level is decreased in E6 co-expressed lanes while it is unchanged in vector co-expressed lanes (Figure2). This result shows that HPVE6 can destabilize Tip60 in breast epithelial cells.

Task3: Addition of MG132 to test whether the degradation of Tip60 is proteasome mediated.

After confirming that HPVE6 can destabilize Tip60 in breast epithelial cells, we hypothesize that this destabilization of Tip60 might be similar to its degradation in keratinocytes by proteasomal pathway. To test this hypothesis we cotransfected the MCF10A cells with Myc-Tip60 alone or with HPVE6 expressing plasmids. Cells were cultured for 48 hrs before harvesting. One set of cotransfected cells were treated with proteasome inhibitor MG132 for 6 hrs before harvesting. Cells were lysed in lysis buffer and equal amount of proteins were loaded for each sample for Western blotting. Western blotting by myc antibody showed that Tip60 level was decreased in samples where it was coexpressed along with HPVE6 (Figure3). MG132 treated samples did not show any decrease in Tip60 levels even in the presence of E6 (Figure3). Western blot of actin showed the equal loading of proteins in all the lanes (Figures3).

This result shows that Tip60 is degraded by proteasome mediated pathway by HPVE6 in breast epithelial cells. Following these experiments, our next task will be to investigate the E3 liagse involved in Tip60 degradation by HPVE6 in breast epithelial cells. By RNAi strategy we will knockdown various E3 ligases and try to see the stabilization of Tip60 in E6 expressing cells.

Task4: Invitro polyubiquitination of Tip60 by E6.

Polyubiqutination is major posttranslational modification on the proteins destined for degradation by proteasomal pathway. After establishing that Tip60 is degraded by E6 through proteasomal pathway we wanted to examine whether this degradation of Tip60

is through its polyubiquitination. Cells were transfected with Myc-Tip60 and Flag-E6 overexpressing plasmids or along with HA-Ub expressing plasmid. Cells were harvested and lysed in HOT lysis buffer. Tip60 was immunoprecipitated from all the 4 samples- 1) mock transfected cells, 2) Myc-Tip60 transfected cells, 3) Myc-Tip60 and E6 cotransfected cells, 4) Myc-Tip60, E6 and HA-Ub transfected cells. Equal amount of immunoprecipitate was run on the polyacrylamide gel for Western blotting by Ubiquitin antibody. The Western result showed that there was not increase in Ubiquitination of Tip60 by the presence of E6 or E6 along with Ub.

This shows that proteasome dependent degradation of Tip60 by E6 does not involve polyubiquitination. We hypothesize that the main function of E6 could be to enhance the recruitment of Tip60 to E3 ligase responsible for its degradation.

Task5: Overexpression of nondegradable Tip60 to see its effect on immortalization of MECs by HPV E6.

After establishing the degradation of Tip60 by E6, our next task was to explore the biological significance of this degradation. For it we try to adopt the strategy of overexpressing Tip60 and making E6 degradation resistant Tip60. To make the Tip60 overexpressing stable cells lines we cloned the Tip60 gene in reterovirus plasmids and made the stable cell lines in breast epithelial MCF10A cells that expresses Tip60 wild or Tip60 HAT mutant proteins (Figure4A). These cell lines will help us in studying the effect of Tip60 and its HAT activity on HPV virus life cycle. In another approach to generate HPVE6 degradation resistant Tip60 we made several deletion constructs of Tip60 and overexpresses them with E6 to check their stability. We identified two regions in Tip60 which seems to be essential for its degradation by Tip60 (Figure4B). Unfortunately when we made the Tip60 deletion construct with those two regions, we failed to see the expression of that mutated Tip60. It seems that these two regions of Tip60 are essential for its protein stability and HPVE6 protein utilizes those motifs to interact and promote its degradation. After narrowing down of the deletion motifs we will now use point mutation approach to narrow down on the essential amino acids in that region that might be essential for its degradation. We hope that these point mutants of Tip60 will solve our expression problem. Once we will get the HPVE6 degradation resistant mutant we will make the stable cell line of it and try to see its effect on HPV in breast epithelial and cancerous cells.

Does Adenovirus degrades Tip60 in breast epithelial cells

Task6: Adenovirus degrades Tip60

In our supplementary aim we checked the effect of another dsDNA virus Adenovirus that also infect humans and causes upper respiratory tract infection and is known to cause tumor in newborn rats. To check the effect of adenovirus on Tip60 levels, MCF10A breast epithelial cells were infected with *dl309* virus (phenotypically wild type adenovirus). Note that multiple splice-isoforms of Tip60 are expressed at varying ratios in different cell-lines. Tip60 levels were decreased in the virus (dl309)-infected MCF10A cells compared to mock-infected cells while the levels of another HAT protein MOF was not decreased (Figure 5A). Evaluating MCF10A lysates harvested at various times post-infection showed that both p53 and Tip60 decreased at about the same time: 64 hr post infection (Figure 5B). The time of decrease corresponded with significant expression of EIB55K and E4orf6 oncoprotein (Figure 5B). The half life of Tip60 was determined by shutting off new protein synthesis by cycloheximide. The half life decreased to <30 min in virus (dl309)-infected cells compared to >120 min in mock-infected cells (Figure 5C). These data suggest that adenovirus infection destabilizes cellular Tip60 protein.

Task7: Tip60 represses the transcription of EIA; major transcription factor of virus

Since Tip60 is a HAT protein and can act as a coregulator for gene transcription we hypothesized that degradation of Tip60 might promote viral gene expression. We therefore checked the expression profiles of different viral genes in cells with wild type Tip60 compared to those with siRNA mediated Tip60-depletion. Tip60 is degraded in *dl309* infected cells around 62 hr post-infection, consequently any difference in viral gene expression due to the experimental knockdown of Tip60 is expected to be transient, before the virus naturally directs degradation of Tip60. Expression of all the early genes was increased in siTip60 cells at early times of infection (Figure 6A). Comparing viral gene expression in siTip60 cells to that in siGL2 cells shows that, EIA expression increased by 3-4 fold in Tip60 depleted cells, while the other viral genes showed at most a 2-fold increase in expression (Figure 6B). The E1A protein was also increased in siTip60 cells. However, expression of all viral genes became comparable in control and siTip60 cells at 96 hr post infection when the virus degrades Tip60, consistent with our expectation. These results show that the presence of Tip60 suppresses the EIA mRNA level early in viral infection.

Task8: Tip60 inhibits Virus DNA synthesis

Viral DNA synthesis is dependent on early viral gene expression. Tip60 represses the master regulator of early viral transcription, EIA. Therefore we hypothesized that adenovirus degrades Tip60 because the latter has a negative effect on viral DNA

replication. To test this hypothesis, cells transfected with siGL2 or siTip60 were infected with the wild type dl309 virus to determine if diminishing the levels of Tip60 at early times of viral infection leads to greater viral DNA replication. Indeed, Tip60 knockdown resulted in greater viral DNA levels at 72 hr post infection (Figure 7A). These results suggest that Tip60 can suppress virus life cycle.

To ascertain whether the lysine acetyltransferase activity of Tip60 has any role in the suppression of viral DNA replication, we made cell lines stably transfected with either empty vector or with plasmids expressing wild Tip60 or catalytically dead Tip60. These cells were then infected with *dl309* strain and harvested at 72 hrs post infection. The cells expressing wild type Tip60 had less viral DNA in comparison to cells expressing catalytically dead Tip60 or transfected with empty vector (Figure 7B). These results show that the acetyltransferase activity of Tip60 suppresses viral DNA synthesis.

Task9: Depletion of Tip60 by Adenovirus modulates cellular genes expression

Tip60 acetylates Histones and changes the epigenetic environment of promoters to change the gene expression profile. We hypothesized that Adenovirus-dependent degradation of Tip60 might also affect expression of cellular targets of Tip60. Microarray analysis after Tip60 knockdown in HCT116 cells revealed several genes to be regulated by Tip60 (Dutta and Dutta, unpublished data). Among these genes Vav-3 and E2F-3 were downregulated by Tip60 knockdown or after dl309 infection (Figure8A). The decrease in expression of both genes coincides with the time of degradation of Tip60 in virus-infected cells: 64 hrs post infection (Figure8B). These results suggest that the decrease in expression of Vav-3 and E2F-3 in dl309 virus infected cells may be dependent on Tip60 degradation.

To verify this hypothesis, we measured the expression of both genes in dl309 virus-infected cells that were stably infected with empty vector or with vector overexpressing Tip60. Overexpression of Tip60 partially alleviated the repression of Vav-3 and E2F-3 seen in adenovirus-infected cells (Figure8C). These results suggest that the degradation of Tip60 by adenovirus results in repression of two cellular genes that are normally up-regulated by Tip60.

Key accomplishments:

-Generated monoclonal antibody against Tip60.

-Generated reagents for Tip60 and HPVE6, like mammalian overexpressing plasmids, RNAi oligos.

-Performed the cotransfection experiment in mammary epithelial cells and establish E6 dependent Tip60 degradation in breast epithelial cells.

-Indentify proteasome pathway dependent degradation of Tip60 by HPVE6.

-Establish that E6 depedent Tip60 degradation

-Generated Tip60 wild and Tip60 HAT mutant overexpressing cell line in breast epithelial cells.

-Confirmed Adenovirus dependent Tip60 degradation. Identify viral proteins involved in this degradation.

-Establish role of Tip60 in inhibiting transcription of major viral transcription factor EIA and viral DNA synthesis.

-Identify the cellular genes expression modulation by adenovirus infection

Reportable Outcomes

1) Paper accepted in Oncogene (In press, doi: 10.1038/onc.2012.534)

"Tip60 Degradation by Adenovirus Relieves Transcriptional Repression of Viral Transcriptional Activator EIA "

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2) Paper accepted in Molecular and Cellular Biology (In press, MCB.01567-12)

"RVBs are required for functional TIP60 complex"

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Conclusions:

HPV and Tip60 modulation in Breast Cancer

In this study we have examined the modulation of Tip60 in breast epithelial cells in the presence of Human papilloma virus oncoprotein E6. Our preliminary results show that E6 can destabilize Tip60 in breast epithelial cells. This destabilization could have been at mRNA level or protein level. To check whether this destabilization is due to protein degradation of Tip60 we blocked the proteasome pathway by MG132 (inhibitor of proteasome machinery). Stabilization of Tip60 by MG132 in of HPVE6 shows that depletion of Tip60 is due to its proteasome dependent degradation. These results are positive sign for the future of the project. After confirming this Tip60 degradation, we will screen for E3 ligase that is involved in its degradation. Blocking or knockdown of that E3 ligase will help in stabilizing Tip60 in HPV infected breast epithelial or cancer cells which can lead us in understanding the role of Tip60 in infected cells and its effects on HPV. Another approach of it is to make degradation resistant Tip60. We have mapped some regions that seem to be essential for Tip60 degradation by E6. Overexpression of this non-degradable Tip60 will help us in deciphering the role of Tip60 on Human papilloma virus and cellular modifications after Tip60 degradation. Our results can lead us in understanding the transformation of breast epithelial cells by HPV and can provide new therapeutic targets that can prevent virus induced breast epithelial cells transformation or breast cancer.

Determining the role of Adenovirus in destabilizing Tip60 and Breast Cancer

Additionally we have studied another dsDNA virus Adenovirus role in modulation of Tip60 in breast epithelial cells. Adenoviruses have developed various strategies to neutralize the host defense system. In this study we have identified that adenovirus can degrade Tip60 in breast epithelial cells. This degradation of the lysine acetyl transferase Tip60 relieves transcriptional repression of the viral major early gene, EIA that is a master transcription factor for the virus, providing a rationale for why the virus evolved a mechanism to degrade Tip60. We have also shown that Tip60 HAT activity inhibits viral DNA synthesis in breast epithelial cells. Adenovirus by depleting Tip60 can modulate the expression of Tip60 targeted genes. Although there is no evidence of the presence of adenovirus in human cancer till now there are reports that suggest a transient requirement of the virus for cell-transformation, following which the cells lose the virus: the so-called Hit-and-run oncogenesis. Increasing evidence suggests that viral oncoproteins can target epigenetic regulators to alter epigenetic regulation of the cells. Tip60 is an important epigenetic factor. Our study shows that adenovirus can target Tip60 in breast epithelial cells and shows that these cells are vulnerable for virus

infection. This viral infection may directly or indirectly augment the transformation and can subsequently lead to breast cancer. Although further studies are required for the conclusive evidences on the role of adenovirus in breast cancer but our results show that our study is on right track.

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Figure1

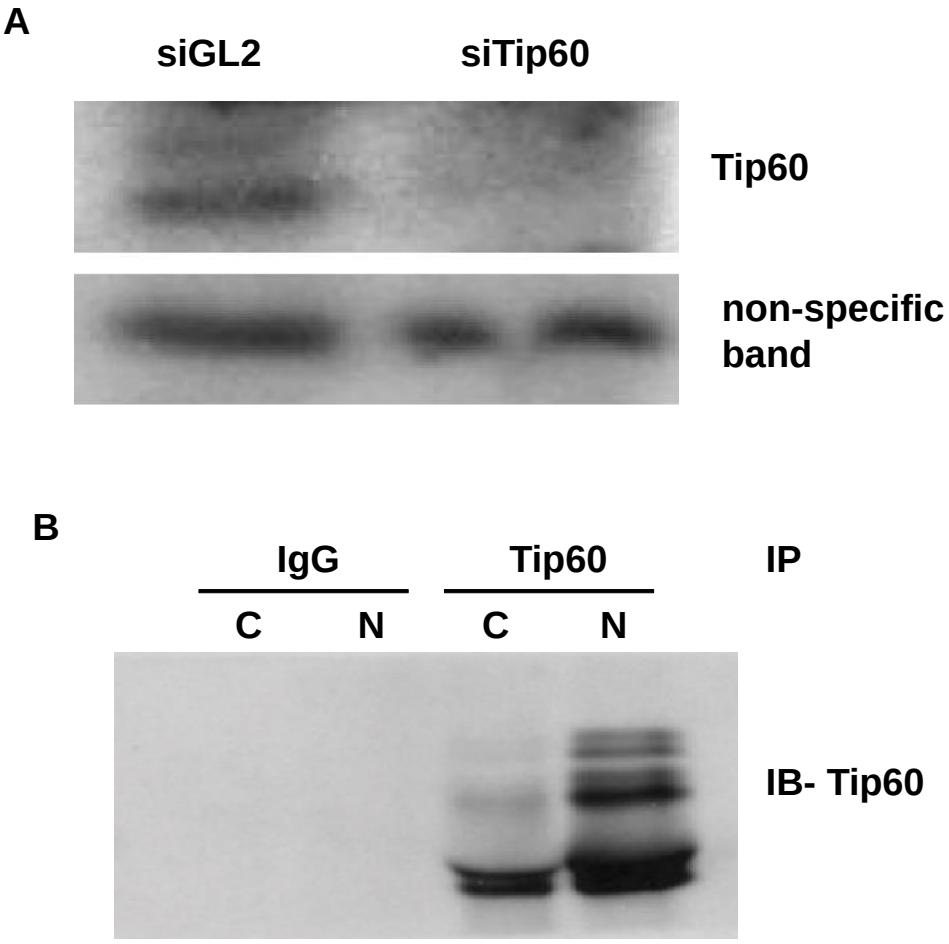
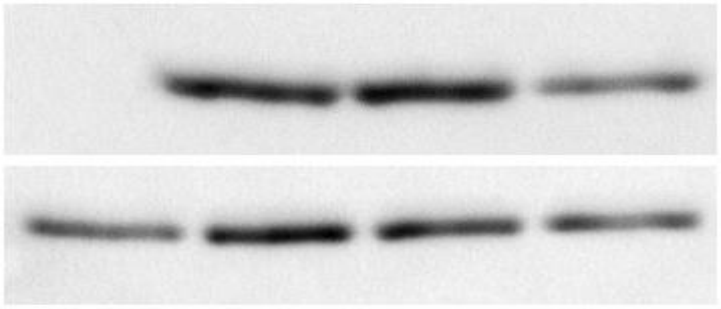
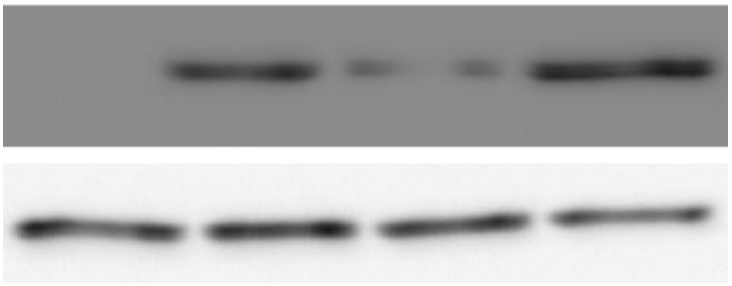


Figure2



Myc/Tip60	-	+	+	+
pCNDA3-Flag/E6	-	-	-	+
pCDNA3	-	-	+	-

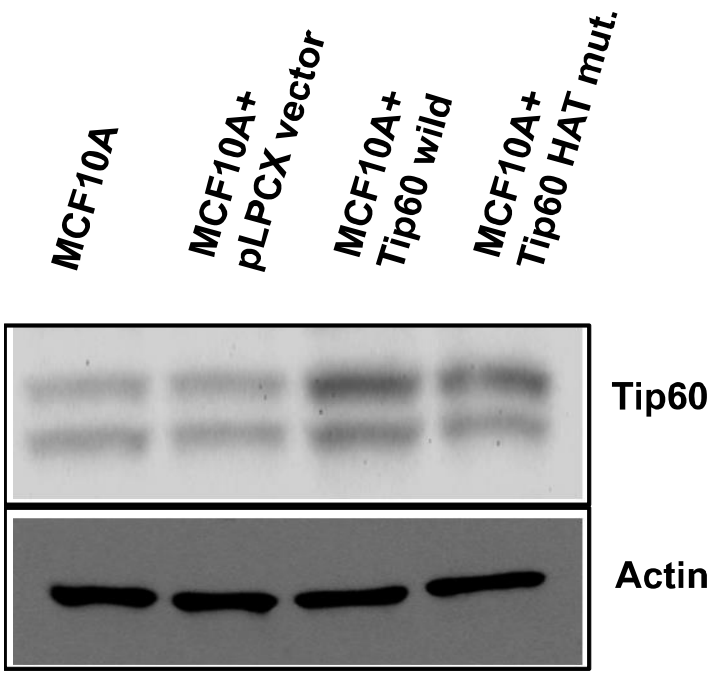
Figure3



Myc/Tip60	-	+	+	+
pCNDA3-Flag/E6	-	-	+	+
MG132	-	-	-	+

Figure4

A



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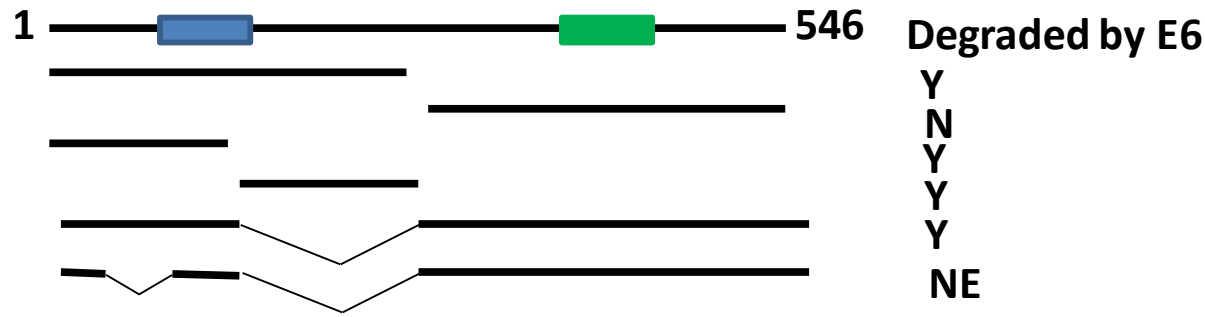


Chromodomain (65-101)

Acetyl CoA domain (398-422)

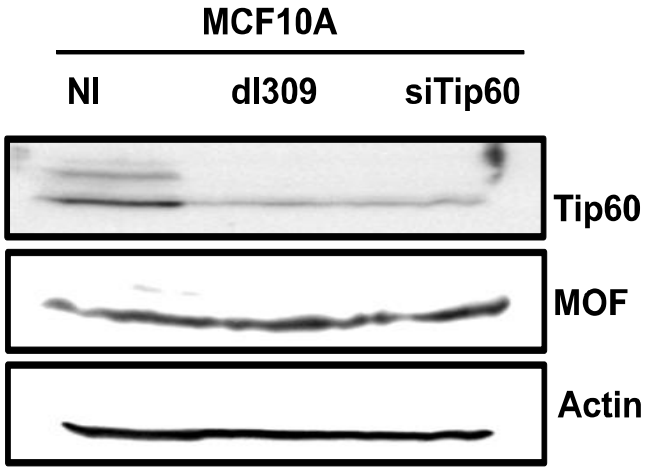
Y Yes

N No

NE Not expressed

Figure5

A


MCF10A
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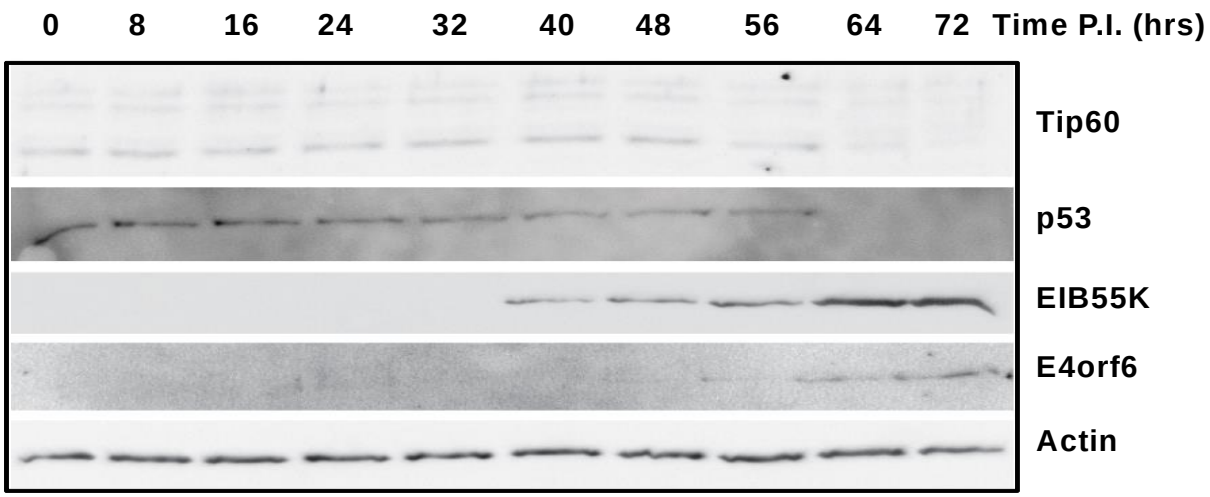
dl309
siTip60
Tip60
MOF
Actin

B


0
8
16
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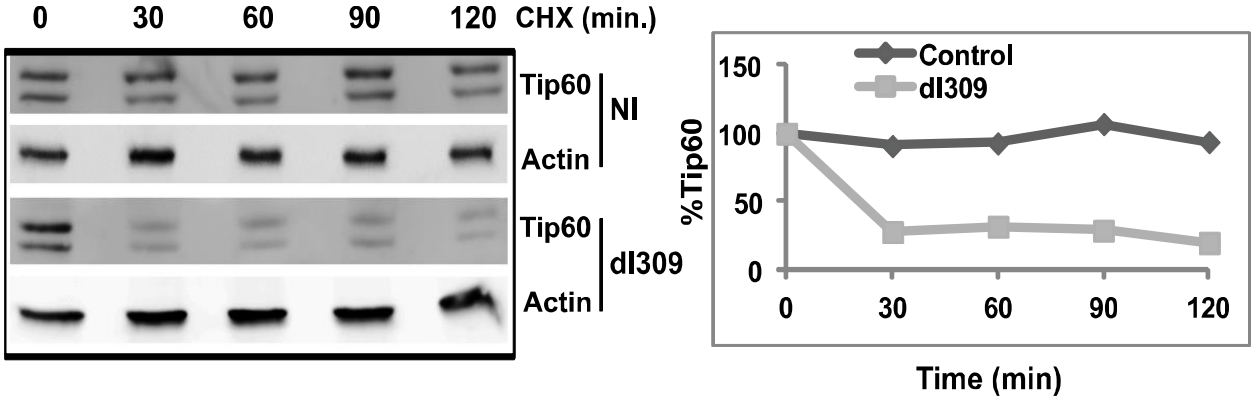
48
56
64
72
Time P.I. (hrs)
Tip60
p53
EIB55K
E4orf6
Actin

C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30
60
90
120
CHX (min.)
Tip60
Actin
NI
Tip60
Actin
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dl309
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100
50
0
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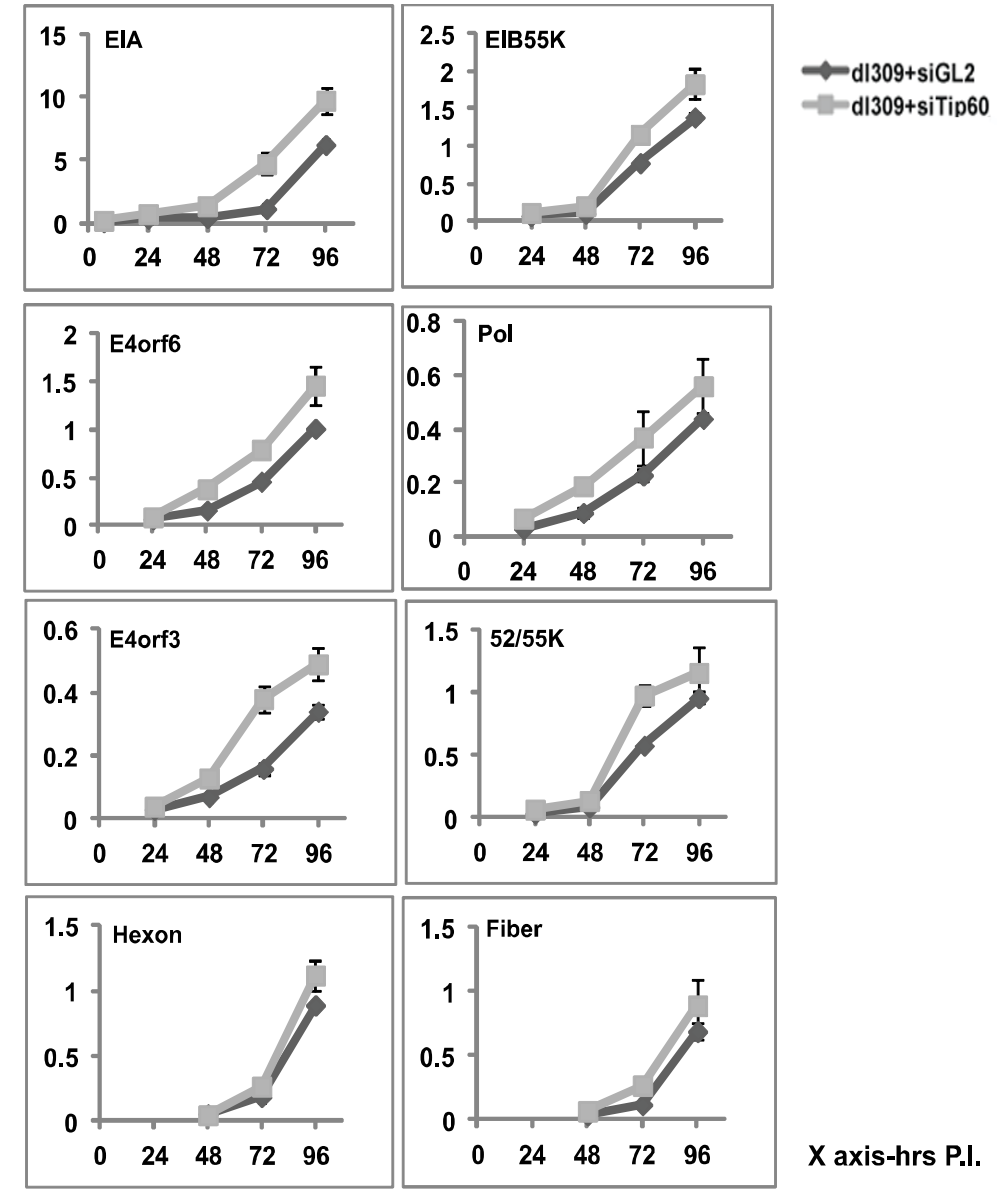
30
60
90
120
Time (min)

Figure6


EIA
EIB55K
E4orf6
Pol
E4orf3
52/55K
Hexon
Fiber
dl309+siGL2
dl309+siTip60
X axis-hrs P.I.

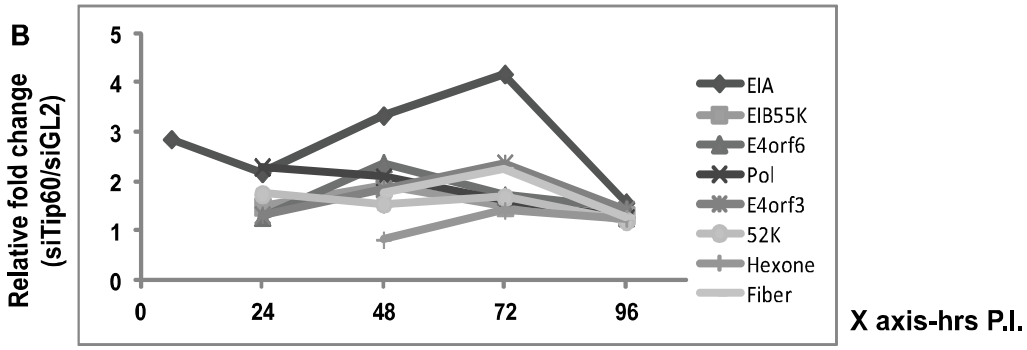
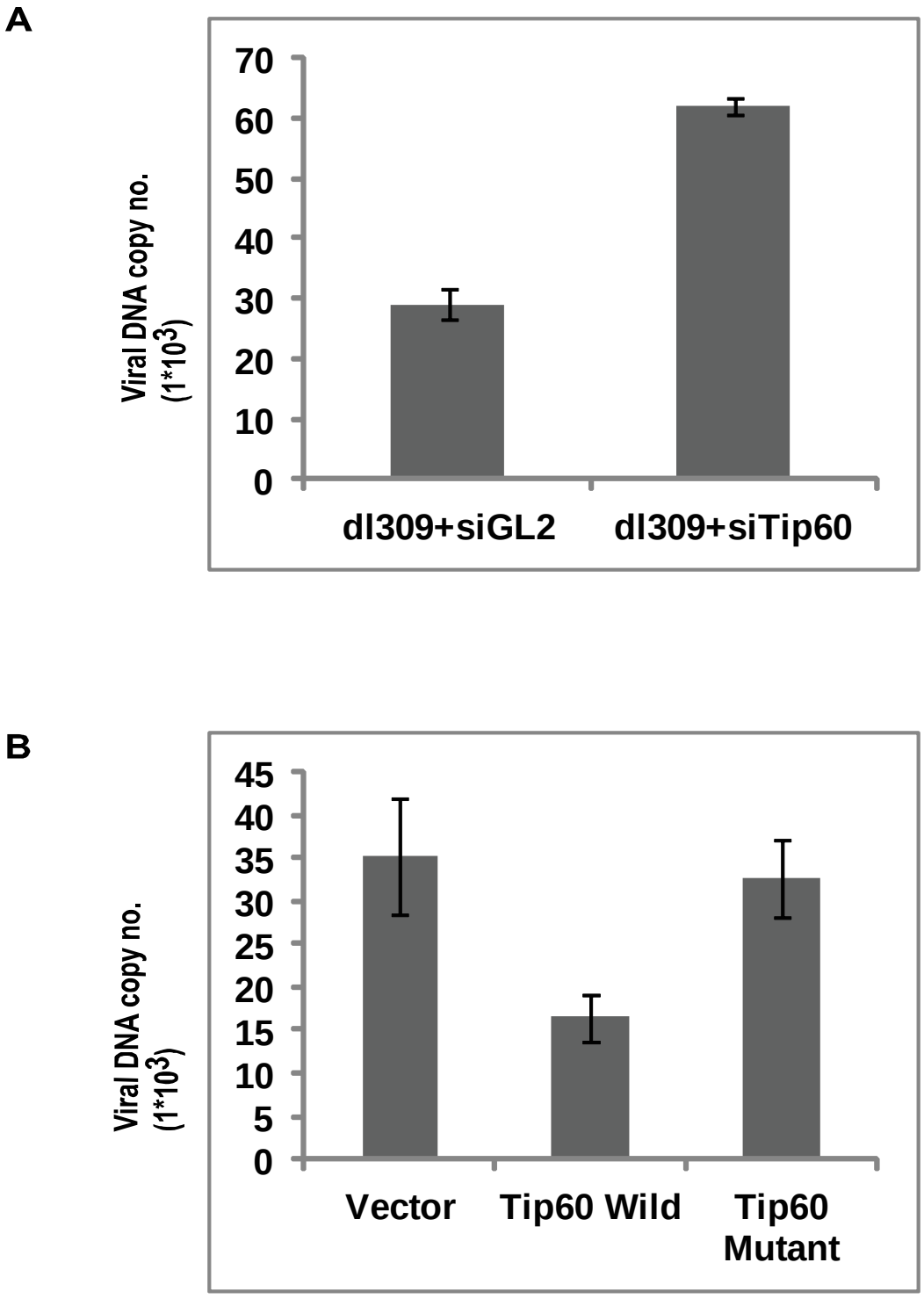

B
Relative fold change (siTip60/siGL2)
EIA
EIB55K
E4orf6
Pol
E4orf3
52K
Hexone
Fiber
X axis-hrs P.I.

Figure7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Viral DNA copy no.
(1*10^3)
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dl309+siTip60
B
45
40
35
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20
15
10
5
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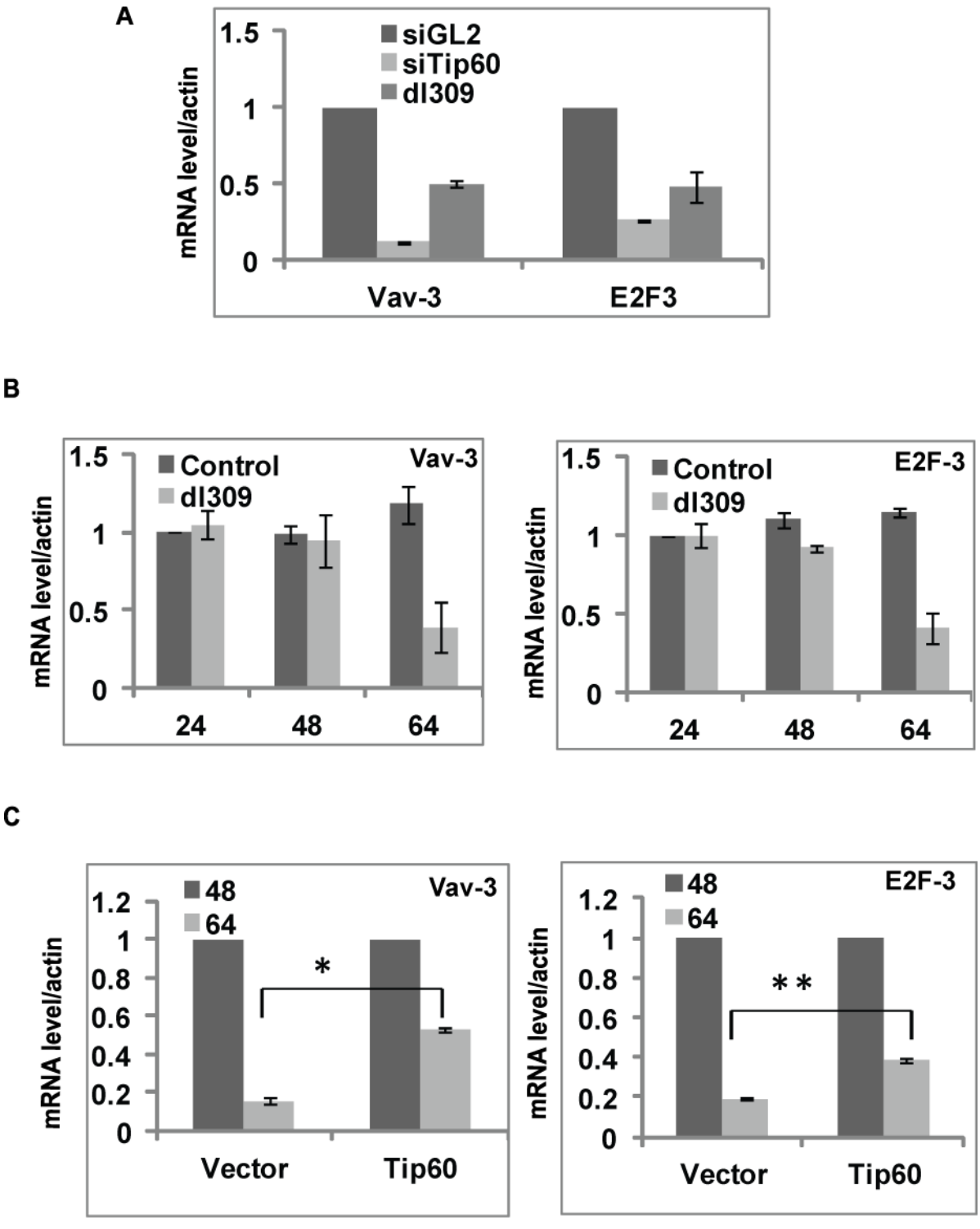
Vector
Tip60 Wild
Tip60
Mutant

Figure8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mRNA level/actin
1.5
1
0.5
0
siGL2
siTip60
dl309
Vav-3
E2F3
B
mRNA level/actin
Control
dl309
Vav-3
24
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Control
dl309
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Vector
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Appendices:

Oncogene- ONC-2012-01332R

Tip60 Degradation by Adenovirus Relieves Transcriptional Repression of Viral Transcriptional Activator EIA

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Running title- Degradation of Tip60 by Adenovirus

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Abstract

Adenoviruses are linear double stranded DNA viruses that infect human and rodent cell lines, occasionally transform them and cause tumors in animal models. The host cell challenges the virus in multifaceted ways to restrain viral gene expression and DNA replication, and sometimes even eliminates the infected cells by programmed cell death. To combat these challenges, adenoviruses abrogate the cellular DNA damage response pathway. Tip60 is a lysine acetyltransferase that acetylates histones and other proteins to regulate gene expression, DNA damage response, apoptosis and cell cycle regulation. Tip60 is a *bona fide* tumor suppressor since mice that are haploid for Tip60 are predisposed to tumors. We have discovered that Tip60 is degraded by adenovirus oncoproteins EIB55K and E4orf6 by a proteasome-mediated pathway. Tip60 binds to the immediate early adenovirus promoter and suppresses adenovirus *EIA* gene expression, which is a master regulator of adenovirus transcription, at least partly through retention of the virally encoded repressor pVII on this promoter. Thus degradation of Tip60 by the adenoviral early proteins is important for efficient viral early gene transcription and for changes in expression of cellular genes.

Key words: HAT; Tip60; EIA; Adenovirus

Introduction

Adenoviruses are non-enveloped, linear, double stranded DNA viruses. Discovered in early 1950s, almost 50 human adenoviruses clustered in 6 different species have been identified. Besides lytic infection, adenoviruses are also known to transform host cells through their oncogenes EIA and EIB in cooperation with other cellular proteins.[1] Although there is no concrete evidence that adenoviruses are involved in tumor formation in humans they can transform human cells and can induce tumors in newborn rats. Adenovirus has developed different strategies to counter host cell defense and usurp the cellular machinery, both for viral replication and cell transformation. Indeed several paradigms of how viral oncoproteins transform cells by inactivating cellular tumor suppressors were elucidated in cells transformed by adenovirus. For example, adenovirus EIA protein binds and inactivates Rb protein to promote entry of cells into S phase, while EIB55K and E4orf6 oncoproteins bind and direct p53 for degradation by proteasome to prevent cell-cycle checkpoint activation and apoptosis. [2-4] Adenovirus also degrades MRN complex proteins, DNA ligase IV, TOPBP1 and integrin alpha 3 (ref.5-8).

Many studies have shown widespread changes in DNA and histone modifications during tumorigenesis.[9] Several viral oncoproteins interact with different histone modifying enzymes of the host cell and deregulate the epigenetic program.[10,11] Recently we showed that the Human Papillomavirus (HPV) oncoprotein E6 interacts

with and destabilizes the cellular histone acetyltransferase protein Tat interacting protein 60 kDa, Tip60 (ref.12). Intriguingly, the degradation of Tip60 relieved cellular repression of the viral early promoter. Another recent study showed that human cytomegalovirus (HCMV) pUL27 can induce Tip60 degradation by proteasome, though the contribution of this degradation to the viral life cycle was unclear. [13]

Tip60 is a lysine acetyltransferase protein of the MYST family. It modifies the chromatin structure by acetylating histones and thus plays an important role in transcriptional regulation.[14-16] Tip60 is essential for viability since its disruption causes embryonic lethality.[17] Mice that lack one allele of Tip60 show acceleration in tumorigenesis.[18] Tip60 has also been implicated in the DNA damage response: it activates the apex kinases in the checkpoint pathways, the ATM/ ATR kinases and it stops the DNA damage response by promoting the dephosphorylation of phosphoH2AX at the DNA damage sites.[19-20] Another non-histone protein acetylated by Tip60 is p53. Tip60 acetylates p53 on lysine120 and shifts the balance towards apoptosis and away from cell-cycle arrest. [21-22]

Tumor suppressors like p53 and Rb are inactivated by HPV and adenovirus oncoproteins. Since Tip60 is a tumor suppressor that is targeted for degradation through proteasome mediated pathway by the HPV E6 oncoprotein, we investigated whether Tip60 was also degraded by adenovirus oncoproteins. We discovered that the adenovirus oncoproteins EIB55K and E4orf6 also direct Tip60 for degradation in a proteasome-dependent manner and by doing so they relieve the repression imposed by cellular Tip60 on viral early gene expression. The results highlight a possible role for

Tip60 during adenovirus replicative cycle, and suggest that downregulation of the tumor suppressor Tip60 may be important for multiple oncogenic viruses.

Results:

Adenovirus destabilizes Tip60 protein

To check the effect of adenovirus on Tip60 levels, MCF10A breast epithelial cells were infected with *dl*309 virus (phenotypically wild type adenovirus). Note that multiple splice-isoforms of Tip60 are expressed at varying ratios in different cell-lines. Tip60 levels were decreased in the virus (*dl*309)-infected MCF10A cells compared to mock-infected cells while the levels of another HAT protein MOF was not decreased (Figure 1A). Similar results were obtained in U2OS osteosarcoma cells and HCT116 colon cancer cells (Figure 1B). Evaluating MCF10A lysates harvested at various times post-infection showed that both p53 and Tip60 decreased at about the same time: 64 hr post infection (Figure 1C). The time of decrease corresponded with significant expression of EIB55K and E4orf6 oncoprotein (Figure 1C). The half life of Tip60 was determined by shutting off new protein synthesis by cycloheximide. The half life decreased to <30 min in virus (*dl*309)-infected cells compared to >120 min in mock-infected cells (Figure 1D). These data suggest that adenovirus infection destabilizes cellular Tip60 protein.

EIB55K and E4orf6 oncoproteins are involved in proteasomal mediated degradation of Tip60

To test whether the oncoproteins of adenovirus are involved in the destabilization of Tip60, we tested different mutants of adenovirus under similar conditions. *dl366** virus with a deletion of the entire E4 region did not affect Tip60 levels, similar to *dl355* virus with a mutation specifically in the E4orf6 gene. *dl1520* virus with a deletion in EIB55K gene also did not decrease Tip60 (Figure 2A). These results suggest the involvement of EIB55K and E4orf6 proteins in the destabilization of Tip60. To confirm this result and to rule out a role of other viral proteins, a plasmid expressing HA/flag-Tip60 was co-transfected with plasmids expressing EIB55K or E4orf6 singly or in combination into U2OS cells. A co-transfected plasmid expressing GST serves as a transfection control. Tip60 levels were reduced in the cells with both EIB55K and E4orf6 but not with either oncoprotein on its own (Figure 2B).

To test whether the destabilization of Tip60 is due to degradation by proteasomes, *dl*309-infected cells were treated with the proteasomal inhibitor MG132. Tip60 decrease was abrogated by MG132 (Figure 2C) implicating proteasomes in the Tip60 degradation. MLN4924 is a global inhibitor of all cullin Ring E3 ligases, because it inhibits the critical neddylation of cullins that is essential for their activity. Treatment with MLN4924 also prevented adenovirus mediated decrease in Tip60 (Figure 2C). Similarly addition of MG132 and MLN4924 prevented the destabilization of transfected HA-Tip60 by co-transfected EIB55K and E4orf6 expression (Figure 2D).

Cullin-5 and the elongins are known to co-operate with E1B55K and E4orf6 to degrade p53 [4]. In order to determine which cullin was involved in the adenovirus-mediated degradation of Tip60, we knocked down individual cullin proteins by siRNA transfection in *dl*309 virus-infected cells. While p53 was stabilized, as expected, by si-

Cullin-5, Tip60 was not stabilized (Figure S1). si-Cullin3 stabilizes Tip60, but since Cullin-3 is known to degrade Tip60 even in uninfected cells ,[23] we cannot be certain that Cullin-3 is specifically recruited by EIB55K+E4orf6 to degrade Tip60. To ascertain whether the elongins may be involved in the degradation of Tip60, we knocked down elongin B by siRNA in *dl*309 virus-infected cells. Knockdown of elongin B stabilized p53 but not Tip60 (data not shown) suggesting that elongins may not be involved in the degradation of Tip60 by E4orf6+EIB55K.

The results suggest that as with p53, the E1B55K+E4orf6 viral proteins may interact with Tip60, to recruit Tip60 to the E3 ligase. To test this, plasmids expressing HA/flag-Tip60 were co-transfected into U2OS cells with plasmids expressing EIB55K and E4orf6 (Figure 2E). Flag/HA-Tip60 was increased in cell lysates when it was stabilized by MG132 (lane 2). Immunoprecipitation of Tip60 from the MG132-treated cells co-immunoprecipitated EIB55K and E4orf6 proteins (lane 4). When E4orf6 or EIB55K were transfected singly with Tip60 and the Tip60 was immunoprecipitated, the viral proteins were co-immunoprecipitated, but to a much lower extent than when they were present together (lanes 7, 9). These results suggest that EIB55K and E4orf6 together form a complex that binds to Tip60 and possibly recruits Tip60 to an unknown cullin.

Tip60 suppresses EIA expression

Since Tip60 is a HAT protein and can act as a coregulator for gene transcription we hypothesized that degradation of Tip60 might promote viral gene expression. We

therefore checked the expression profiles of different viral genes in cells with wild type Tip60 compared to those with siRNA mediated Tip60-depletion. Tip60 is degraded in *dl*309 infected cells around 62 hr post-infection, consequently any difference in viral gene expression due to the experimental knockdown of Tip60 is expected to be transient, before the virus naturally directs degradation of Tip60. Expression of all the early genes was increased in siTip60 cells at early times of infection (Figure 3A). Comparing viral gene expression in siTip60 cells to that in siGL2 cells shows that, EIA expression increased by 3-4 fold in Tip60 depleted cells, while the other viral genes showed at most a 2-fold increase in expression (Figure 3B). The E1A protein was also increased in siTip60 cells (Figure S2A). However, expression of all viral genes became comparable in control and siTip60 cells at 96 hr post infection when the virus degrades Tip60, consistent with our expectation. These results show that the presence of Tip60 suppresses the EIA mRNA level early in viral infection.

The increase in EIA expression after knockdown of Tip60 could be secondary to virus replication. To rule this out, we added hydroxyurea (HU) 48 hrs after infection and harvested the cells at 72 hrs post infection and measured EIA transcript levels. HU did not prevent the increase in EIA expression when Tip60 was knocked down showing that the stimulation of E1A expression by siTIP60 is not secondary to an increased number of templates because of viral DNA replication (Figure S2B).

We next used chromatin Immunoprecipitation (ChIP) experiments to determine if Tip60 was preferentially associated with the EIA compared to the Major Late promoter, which remains unchanged after siTip60. Tip60 was detected at the *EIA* promoter but not at the ML promoter (Figure 4A). A cellular promoter known to be regulated by Tip60,

nucleolin, and another unaffected by Tip60, acetylcholine receptor, served as positive and negative controls, respectively. Primer pairs spanning 6 different regions of the E1A promoter (Figure S3) indicated that more Tip60 binds near the transcriptional start site of EIA (Figure 4B). ChIP for histone H4 acetylation measures Tip60 activity at the promoter. pVII is an adenovirus late protein that is known to bind to E1A promoter, represses it and is removed when transcription of viral DNA starts.[24] Both acetylated histone H4 (H4Ac) and pVII protein were bound to the EIA promoter at 50 hours after infection when Tip60 is not degraded, and both disappear from the promoter at 80 hours after infection when Tip60 is degraded (Figure 4C). The strong correlation of Tip60 presence at the E1A promoter with H4Ac, and with pVII which acts as an anti-correlative marker for transcription synthesis, supports the hypothesis that by binding to the E1A promoter and through the acetylation of histone H4, Tip60 represses the E1A promoter.

To test this hypothesis, we knocked down Tip60 by siRNA and measured the amount of H4Ac and pVII at the EIA promoter at 50 and 80 hr post infection. H4Ac and pVII at the EIA promoter decreased prematurely at 50 hr post infection upon knockdown of Tip60 (Figure 4D). To investigate the role of the acetyltransferase activity of Tip60 in retention of H4Ac at the E1A promoter, the ChIP assays were repeated after virus infections in cells with vector alone or overexpressing Tip60 wild type or Tip60 HAT mutant. The decrease in H4Ac and in pVII at the E1A promoter normally seen at 80 hr post-infection was attenuated by wild type Tip60 but not by the catalytically dead Tip60 (Figure 4E). These results indicate that catalytically active Tip60 acetylates H4 and thus represses the EIA promoter.

To test whether Tip60 can repress the EIA promoter directly, U2OS cells stably over-expressing Tip60 were transiently transfected with a luciferase reporter driven by the EIA promoter (E1ApGL3c). The over-expressed Tip60 significantly repressed the luciferase activity from E1ApGL3c compared to that driven by the control promoter, pGL3c (Figure 5).

Collectively these results suggest that Tip60 binds to the EIA promoter and represses the EIA promoter through acetylation of Histone 4.

Tip60 knockdown favors initial establishment of viral DNA synthesis

Viral DNA synthesis is dependent on early viral gene expression. Tip60 represses the master regulator of early viral transcription, EIA. Therefore we hypothesized that adenovirus degrades Tip60 because the latter has a negative effect on viral DNA replication. To test this hypothesis, cells transfected with siGL2 or siTip60 were infected with the wild type *dl*309 virus to determine if diminishing the levels of Tip60 at early times of viral infection leads to greater viral DNA replication. Indeed, Tip60 knockdown resulted in greater viral DNA levels at 72 hr post infection (Figure 6A). This result suggest that Tip60 can suppress virus life cycle.

To ascertain whether the lysine acetyltransferase activity of Tip60 has any role in the suppression of viral DNA replication, we made cell lines stably transfected with either empty vector or with plasmids expressing wild Tip60 or catalytically dead Tip60 (Figure S4). These cells were then infected with *dl*309 strain and harvested at 72 hrs post infection. The cells expressing wild type Tip60 had less viral DNA in comparison to

cells expressing catalytically dead Tip60 or transfected with empty vector (Figure 6B). These results show that the acetyltransferase activity of Tip60 suppresses viral DNA synthesis.

Tip60 degradation alters expression of cellular genes

Tip60 acetylates Histones and changes the epigenetic environment of promoters to change the gene expression profile. We hypothesized that Adenovirus-dependent degradation of Tip60 might also affect expression of cellular targets of Tip60. Microarray analysis after Tip60 knockdown in HCT116 cells revealed several genes to be regulated by Tip60 (Dutta and Dutta, unpublished data). Among these genes Vav-3 and E2F-3 were downregulated by Tip60 knockdown or after *dl309* infection (Figure7A). The decrease in expression of both genes coincides with the time of degradation of Tip60 in virus-infected cells: 64 hrs post infection (Figure7B). These results suggest that the decrease in expression of Vav-3 and E2F-3 in *dl309* virus infected cells may be dependent on Tip60 degradation.

To verify this hypothesis, we measured the expression of both genes in *dl309* virus-infected cells that were stably infected with empty vector or with vector overexpressing Tip60. Overexpression of Tip60 partially alleviated the repression of Vav-3 and E2F-3 seen in adenovirus-infected cells (Figure7C). These results suggest that the degradation of Tip60 by adenovirus results in repression of two cellular genes that are normally up-regulated by Tip60.

Discussion

We suggest that Tip60-dependent acetylation of H4 at the EIA promoter represses the immediate early viral promoter. Since EIA is critically important for expression of other viral genes, the degradation of Tip60 may promote efficient EIA-dependent viral transcription and regulate expression of cellular genes (Figure 8). While this model offers a novel perspective on how the degradation of Tip60 by the adenovirus E1B55K and E4orf6 affects the course of a productive infection, Tip60 degradation may also have consequences for cell transformation by the adenovirus. Different investigative groups have demonstrated that the EIA protein alters the epigenetic program of the cell. [25-28] EIA binds to the promoters of genes involved in the cell cycle and growth, cause hypoacetylation at H3K18 and thus stimulates the cell cycle. EIA also binds to the promoters of antiviral genes and differentiation genes and causes their transcriptional repression. Thus the degradation of Tip60 is expected to affect the expression of several cellular genes through the induction of E1A. Our results suggest that in addition, the degradation of Tip60 directly affect the expression of cellular genes normally regulated by Tip60 (Figure 8).

Adenoviruses can cause tumors in rodents and Syrian hamsters and can transform rodent cells *in vitro*. In fact, this latter property has been successfully utilized to understand how viral oncoproteins like E1A and E1B55K inactivate a variety of cellular tumor suppressors such as Rb and p53. Since Tip60 is a *bona fide* tumor suppressor,[18] and since Tip60 is known to be degraded by at least one other viral oncogene, HPV E6, we suspect that the degradation of Tip60 by adenovirus may also be important for cell transformation.

Although there is little evidence of the presence of adenovirus in human cancer there are reports that suggest a transient requirement of the virus for cell-transformation, following which the cells lose the virus: the so-called Hit-and-run oncogenesis.[29] Increasing evidence suggests that viral oncoproteins can target epigenetic regulators to alter epigenetic regulation of the cells.[11] Tip60 is an important epigenetic factor. By acetylating histone H4, Tip60 alters the expression of many cellular genes. Tip60 is also very important for a cell's response to DNA damage: it activates the checkpoint pathways by activating ATM, [30,31] directs p53 to apoptotic pathways [21,22] and turns off the alarm signal by dephosphorylating phosphoH2AX at the end of the damage response.[20, 32-36] Thus it is conceivable that the degradation of Tip60 by an adenovirus infection predisposes the host cell to deleterious mutations caused by DNA damage (Figure 8). Destruction of Tip60 by an adenovirus infection may thus be part of the "Hit-and-run" method of oncogenesis. Once additional mutations convert a cell to the frankly transformed state, the viral oncoprotein may no longer needed.

The adenovirus oncoproteins EIB55K and E4orf6 are required for the destabilization of Tip60 protein. Chronologically the degradation of Tip60 in adenovirus-infected cells is nearly concurrent to the degradation of p53 protein. E1B55K and E4orf6 are also important for the degradation of p53, and they do so by interacting with p53 and recruiting it to a Cullin-5 complex containing elongins B and C.[4] Our experiments suggest that Tip60 is recruited similarly by E1B55K and E4orf6, perhaps to Cullin-3, but also perhaps to an unknown E3 ligase complex that is inhibited by the neddylation inhibitor MLN4924.

Two studies showed that pVII protein remains associated with viral DNA during the early stages of infection. The start of viral transcription releases the pVII protein from viral genome. [24, 37] Our results extend it to say that Tip60 and H4 acetylation at the adenovirus EIA promoter may be correlated with pVII retention at the promoter. We do not know whether the acetylation of H4 at the promoter or of some other protein at or away from the promoter is responsible for the pVII retention and repression of the promoter. The minimum hypothesis is that the acetylation of H4 by Tip60 at the E1A promoter recruits a cellular repressor complex, similar to the recruitment of the Brd4 complex at the HPV major early promoter,[12] and that this repressor inhibits EIA transcription.

Since Tip60 suppresses EIA expression, which is the major viral transcription factor, we were also interested to see the effect of Tip60 knockdown on viral DNA synthesis. The difference in viral DNA copy number suggests that Tip60 limits the number of viral genomes recruited for DNA replication.

p53 and Rb are major tumor suppressors that are inactivated by diverse oncogenic viruses. Thus it is quite exciting that oncoproteins from at least two different viruses (HPV and adenovirus) inactivate Tip60, another known tumor suppressor. Whether the inactivation of Tip60 by these multiple viruses contributes to viral tumorigenesis or to the viral life cycle are important questions that need to be addressed. It is worth noting that besides HPV E6 and adenovirus E1B55K+E4orf6, some other viral proteins are known to interact with Tip60: pUL27 of CMV, Tat protein of HIV, Herpesvirus conserved kinases and KSHV LANA protein. [13, 38-40] Therefore

degradation of or interaction with Tip60 seems to be important for several types of viruses.

Materials and Methods

Cell culture

MCF10A (non-tumorigenic epithelial cell line) cells were cultured in DMEM- F12 medium containing donor calf serum, while U2OS cells (human osteosarcoma cell line) were maintained in DMEM media containing 10% donor calf serum. HCT116 cells (human colorectal carcinoma cell line) were grown in McCoy media containing 10% fetal bovine serum. Cells were maintained in sub-confluent cultures in a 5% CO_2 atmosphere at 37^{o}C.

Antibodies and Western blotting

For western blotting cells were lysed in IPH buffer (50mM Tris-HCl pH-8, 150mM NaCl, 5mM EDTA, 0.5%NP40, 1mM dithiothreitol, 20mM NaF and protease inhibitor mix (Sigma)) and then subsequently sonicated. Lysates were immunobloted with indicated antibodies. Tip60 antibodies were used as described elsewhere.[12] Other antibodies are as follows: anti-p53 (Santacruz), anti-Flag (Sigma), anti-pVII, [41] anti-EIB55K, [42] E4orf6, [43] anti-GST (Santacruz), anti-β actin (Sigma).

Viruses

The *dl*309 strain is phenotypically wild type Ad5 which lacks a portion of E3 region. The *dl*355 mutant virus contains deletion in E4orf6 region while *dl366** mutant virus has deletion in entire E4 region. Mutant *dl*1520 contains a deletion of 827 bp in the 55kDa protein coding region. [44]

Cells were passaged 16-20 hr before infection to density of $3X10^6$ cells. Cells were washed with phosphate buffer saline (PBS) and then the medium was replaced with medium without serum with virus (MOI 5) and 100U/ml penicillin and 100ug/ml of streptomycin. The cells were gently rocked intermittently and incubated for 120 min at 37^{o}C. The virus suspension was then removed and cells were grown in normal medium at 37^{o}C.

Plasmid construction, Transfection and Stable cell lines

Construction of human Tip60-expressing plasmids is described elsewhere. [20] Wild Tip60 and HAT mutant Tip60 expressing cell line were made in MCF10A cells.

For siRNA Transfection, 30nM of annealed siRNA duplex (Invitrogen) with Lipofectamine 2000 RNAi max reagent (Invitrogen) was used as per manufacturer's instructions. Target sequences of oligonucleotides are provided in Supplemental information.

Real Time PCR and Chromatin Immunoprecipitation assay and Luciferase assay

For measuring viral gene and cellular gene expression RNA was isolated from virus-infected cells by TRIzol reagent (Invitrogen) according to the manufacturer's instructions

(Invitrogen). These cDNAs were used as template for real time PCR using SYBR green PCR master mix (Applied Biosystems). Sequences of primers used are in Supplemental information.

For viral DNA quantitation total DNA was isolated from infected cells and viral DNA was quantitated by qPCR using specific primers from AdenoX qPCR titration kit (Clontech, cat- 632253). ChIP assay was performed as described elsewhere.[12] Primers used for ChIP PCR are given in Supplemental information.

Luciferase assay was performed by transfecting the firefly Luciferase gene driven by control (GL3) or EIA promoter (EIAGL3), along with Renilla Luciferase expressing plasmid as control. Cells were harvested 48 hr after Transfection. Luciferase assay was done following manufacturer's instructions (Promega). The firefly Luciferase activity is normalized to renilla Luciferase. The ratio in pGL3c or EIApGL3c (without Tip60) is held as 1.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank the members of Dutta laboratory for lively discussions and helpful comments. This work was supported by RO1 GM084465 to A.D. A.G. was supported by US army Postdoctoral fellowship for breast cancer research DOD-W81XWH1110687.

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Figure Legends

Figure1. Tip60 is destabilized in adenovirus-infected cells.

(A) Immunoblot with indicated antibodies of MCF10A cells (NI- no infection; *dl*309-infected with *dl*309 adenovirus; siTip60- transfected with siTip60). Cells were harvested 68 hrs post infection. Knockdown of Tip60 confirms the identity of the Tip60 bands.

(B) Destabilization of Tip60 by adenovirus in other cell lines. HCT116 and U2OS cells were lysed 40 hours after infection or 72 hr after siRNA transfection prior to immunoblotting.

(C) Tip60 destabilization is concurrent with p53 degradation in adenovirus-infected cells. *dl*309 infected MCF10A cells were harvested at different time points as indicated after infection and lysates were used to probe with indicated antibodies.

(D) Tip60 protein half life decreases in adenovirus-infected cells. *dl*309-infected and uninfected MCF10A cells were treated with cycloheximide (added at 68hrs post infection) for different time points as indicated and lysates were resolved for Western blot by indicated antibodies. (Graph) Levels of Tip60 were quantitated using Gene Tool Software (Syngene). Tip60 levels in both 0 hours lanes are taken as 100%.

Figure2. Adenovirus oncoproteins EIB55K and E4orf6 promote proteasome-dependent Tip60 degradation.

(A) MCF10A cells were infected with different strains of adenovirus and lysates prepared 72 hours after infection were probed with indicated antibodies.

(B) U2OS cells were transformed with indicated plasmids. Cells were harvested 48 hours after transfection and lysates immunoblotted with indicated antibodies. GST-expressing plasmid was cotransfected as a transfection control.

(C) Inhibition of the proteasome or Cullins activity stabilizes Tip60 in *dl*309-infected cells. MCF10A cells were infected by *dl*309 strain and MG132 or MLN4924 was added

to infected cells at 4 or 24 hours before harvesting (72hrs post infection), respectively. Lysates were probed with indicated antibodies.

(D) U2OS cells were transfected with indicated plasmids and addition of either MG132 or MLN4924 stabilizes the Tip60 protein. Rest is as in (B).

(E) Interaction between Flag/HA-Tip60, EIB55K and E4orf6. U2OS cells were transfected with indicated plasmids for 48 hours and treated for 5 hours with or without MG132 before harvest. Anti-flag antibodies and control IgG were used for immunoprecipitation and immunoprecipitated proteins resolved and probed with indicated antibodies. Immunoblot shows co-precipitation of Flag/HA-Tip60, EIB55K, and E4orf6. Input lanes contain 5% of lysates input into the immunoprecipitates.

Figure3. Tip60 represses expression of viral transcription factor EIA.

(A) Levels of mRNA of different viral genes; EIA (early), EIB55K (early), E4orf6 (early), Pol (early), E4orf3 (early), 52/55K (early/late) Hexone (late) and Fiber (late) were measured at indicated time points after viral infection by RT-qPCR and normalized to β actin. MCF10A cells transfected with siGL2 or siTip60 were infected with *dl*309. Mean ± SD (n=3).

(B) Relative expression of different viral genes in siTip60 transfected cells relative to siGL2 transfected cells. EIA mRNA shows most enhancement at 72 hr after infection in cells where Tip60 was knocked down.

Figure4. Tip60 binds to EIA promoter, acetylates histone H4.

(A) Tip60 binds to EIA promoter. ChIP assay was performed in MCF10A cells 72 hours after infection with *dl*309 strain. Ratio of qPCR signal in Tip60 immunoprecipitate relative to that in pre-immune serum immunoprecipitate shows occupancy of Tip60 on EIA promoter. ML- major late promoter. Nucleolin and AcHR were taken as positive and negative control cellular promoters for Tip60 binding. Mean ± SD (n=3).

(B) Primers covering 6 regions of the (Supp Fig. S3) EIA promoter were used in the ChIP assay for Tip60 as in (A). Tip60 binds more near the start site of the EIA transcript. Mean ± SD, n=3.

(C) Acetylated histone H4 and pVII at the EIA promoter decreases after Tip60 degradation at 62 hr post infection. ChIP analysis at EIA promoter at 50 hr and 80 hrs after infection. The rest is as in (A). Mean ± SD, n=3.

(D) Tip60 knockdown accelerates the decrease of H4 acetylation and pVII occupancy at the EIA promoter. ChIP assay was performed in *dl*309-infected MCF10A cells transfected with control siGL2 or siTip60. Level of acetylated H4 and pVII determined by ChIP assay 50 and 80 hrs after infection. mean ± SD, n=3.

(E) Tip60 HAT activity stimulates H4 acetylation and pVII occupancy at EIA promoter. Relative Q-PCR signal ratio at 80 hrs post infection (50 hrs ratio=1) in cells stably transfected with empty vector or plasmids expressing Tip60 wild type or HAT mutant.

ChIP for acetylated H4 and pVII. Mean ± SD, n=3. (*P<0.005and **P<0.005 by Student's t test).

Figure5. Tip60 represses the EIA promoter.

Tip60 represses EIA promoter. Firefly plasmid pGL3C or EIA promoter/pGL3C plasmids were transfected in control or Tip60 overexpressing U2OS cells. Renilla Luciferase plasmid was cotransfected as normalizing control. The ratio of Luciferase activity of firefly to renilla was calculated and normalized values are shown. Mean ± SD of two experiments (n=2) done in triplicate. * P<0.05 by Student's t test.

Figure6. Tip60 null condition favors viral DNA synthesis.

(A) Tip60 knockdown increases viral DNA production. Viral DNA copy number was measured in MCF10A cells infected with *dl*309 strain and treated with control siGL2 or siTip60 at 72 hrs post infection. Mean ± SD (n=3).

(B) Tip60 HAT activity is required for suppression of viral DNA titer. MCF10A cell line stably over-expressing either wild Tip60 or catalytically dead Tip60 or stably transfected with vector alone were infected with *dl*309 strain and viral DNA copy number was measured at 72 hrs post infection as in (A). Mean ± SD (n=3).

Figure7. Tip60 degradation by adenovirus decreases Tip60 dependent cellular gene expression.

(A) Relative expression of cellular genes in MCF10A cells transfected with siGL2, siTip60 or infected with *dl309*. Cells were harvested 64 hrs post infection or transfection. Relative expression of genes by RT-qPCR compared to control siGL2. Mean ± SD (n=3).

(B) Decrease in cellular gene expression coincides with Tip60 degradation in *dl309*-infected cells. MCF10A cells were infected with mock or dl309 virus and cells were harvested at different time points as indicated. Relative mRNA expression of Vav-3 and E2F-3 was measured at indicated times by RT-qPCR taking value of Control 24 hrs sample as 1. Mean ± SD (n=3).

(C) Tip60 overexpression can rescue decrease in cellular genes expression. Relative mRNA expression of Vav-3 and E2F-3 at 48 and 64 hrs post infection in cells stably transfected with empty vector or Tip60 expressing plasmid. Level of expression at 48 hrs in vector or Tip60 overexpressing cells is taken as 1. Mean ± SD, n=3. (*P<0.05and **P<0.05 by Student's t test).

Figure8. Model for relief of Tip60 suppression of the EIA promoter by early viral genes and resultant viral production.

The EIA promoter in a fraction of the input viral genomes is suppressed by Tip60 (inactive pool). Early gene expression from a limited initial pool of active viral genomes leads to expression of the EIB and E4 genes with concomitant degradation of Tip60

through the proteasome. The loss of Tip60 de-represses the EIA promoter which leads to high levels of transcription, viral DNA replication and productive infection

Figure1

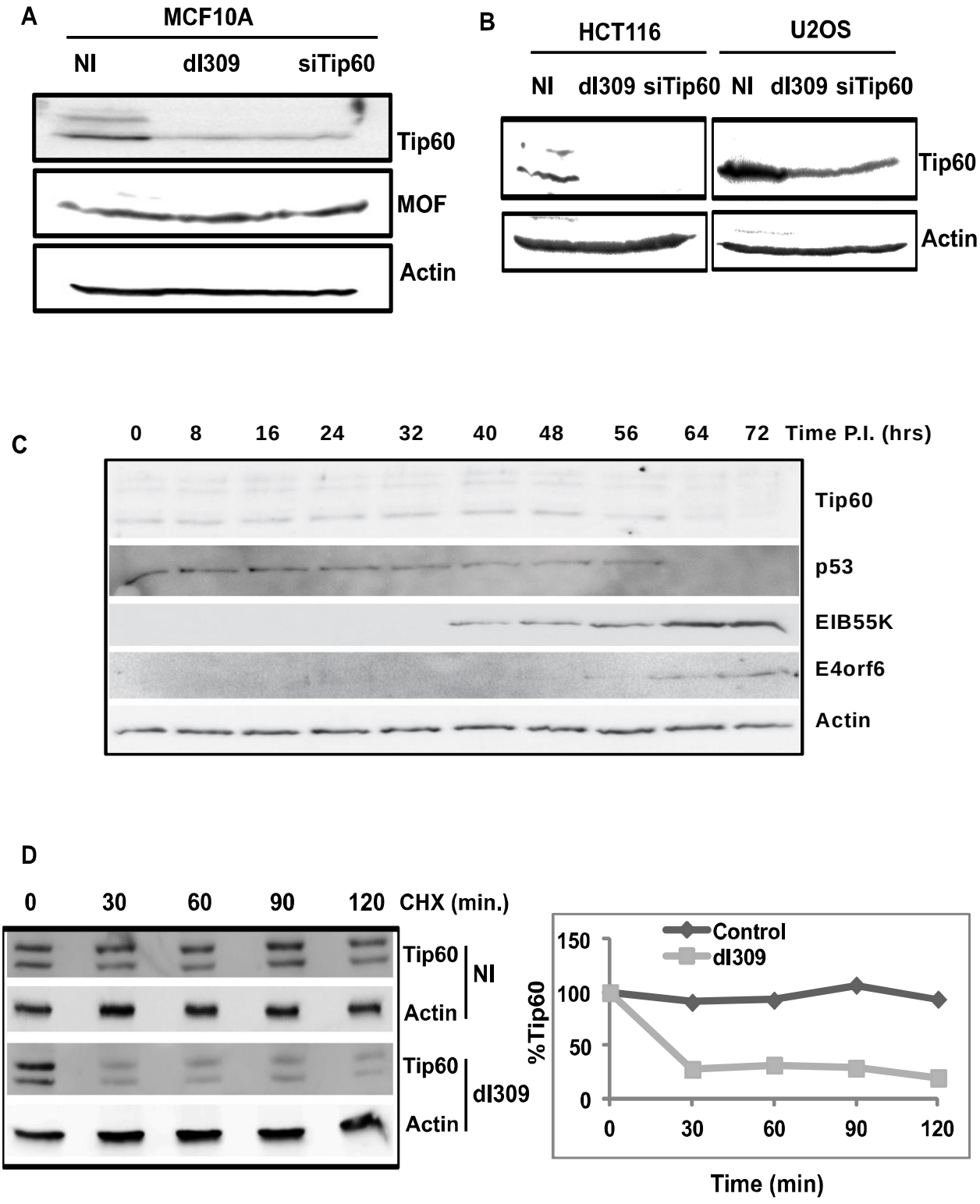
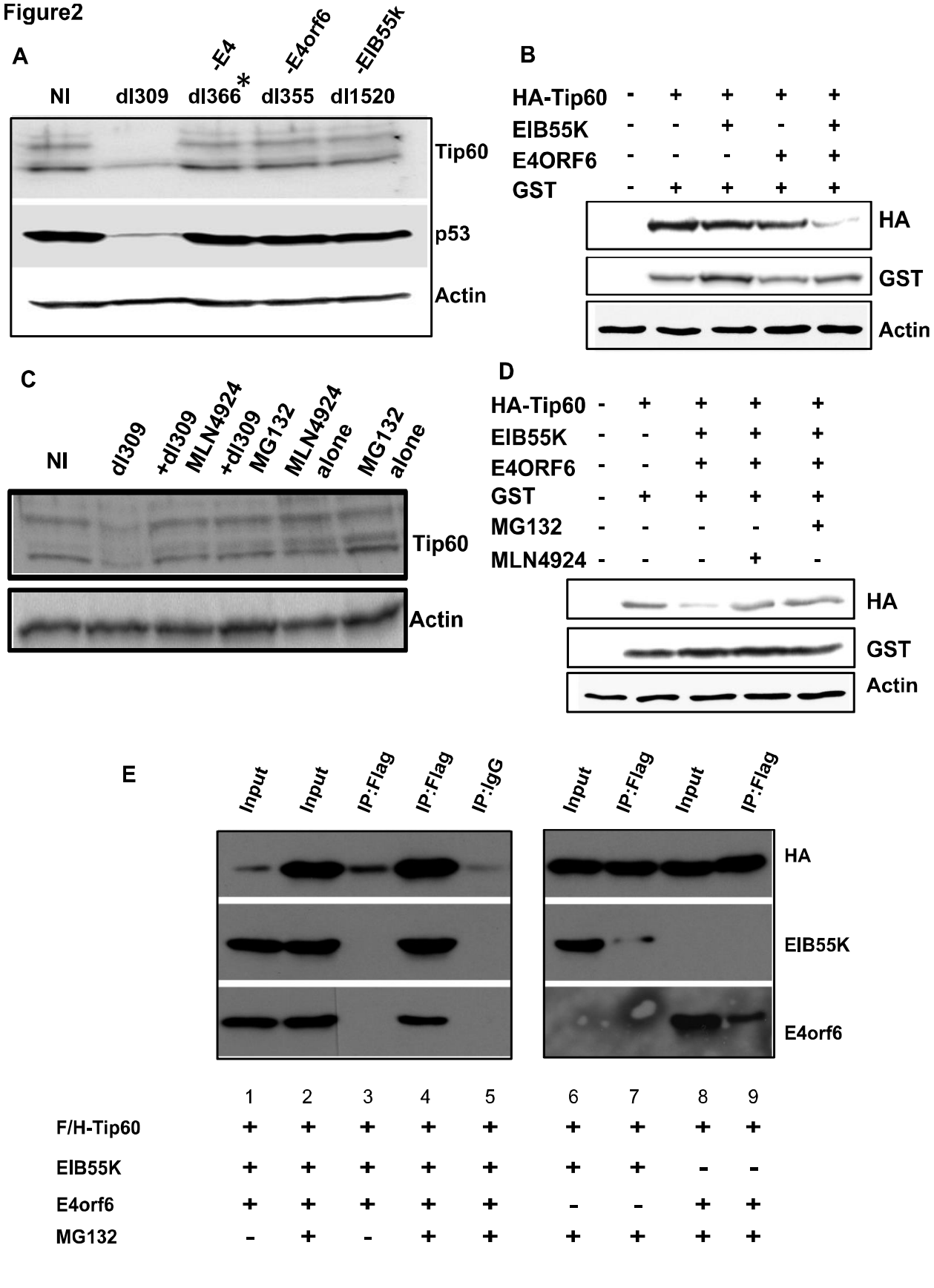
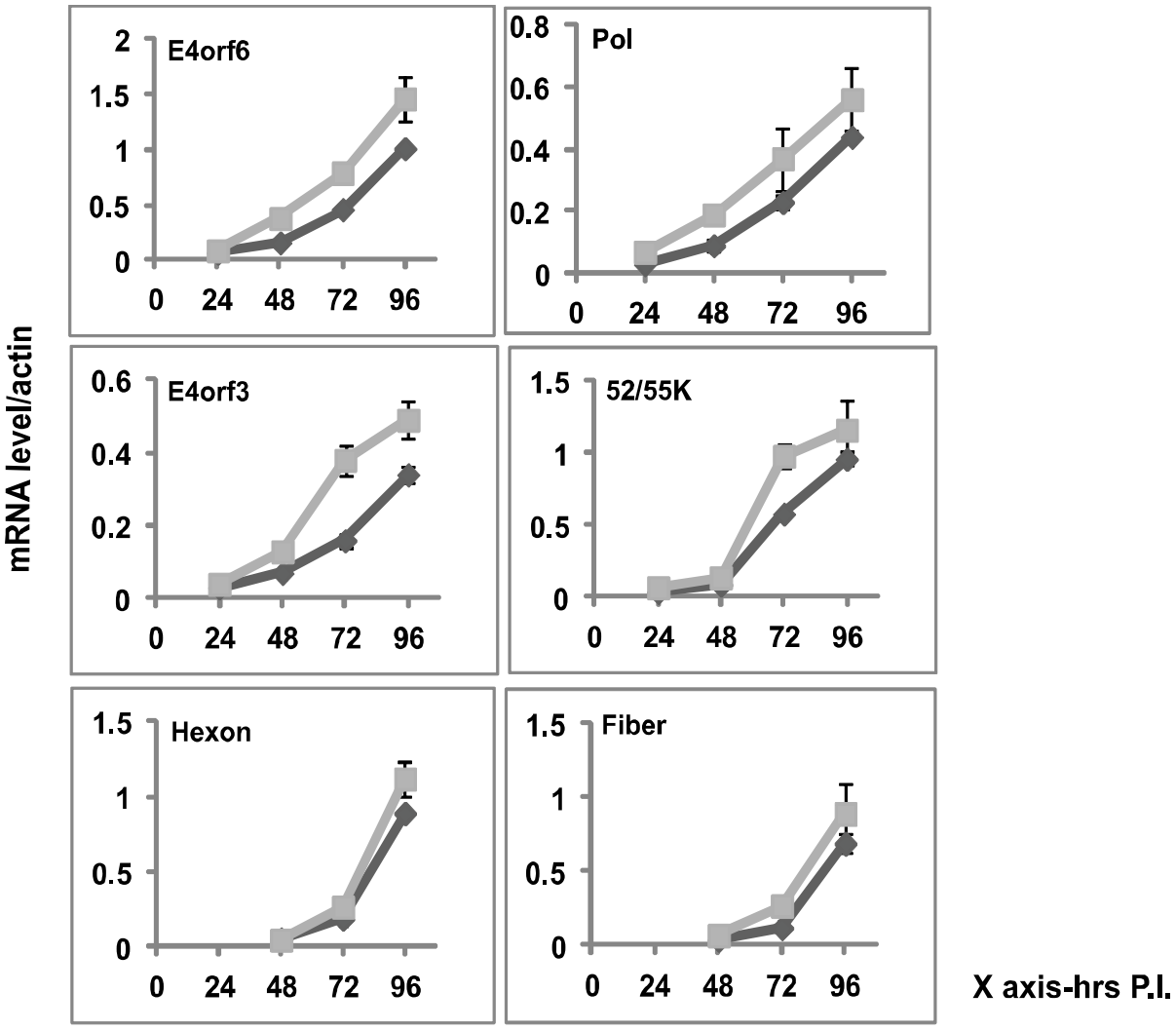

A
MCF10A
NI
dl309
siTip60
Tip60
MOF
Actin
B
HCT116
U2OS
NI
dl309
siTip60
NI
dl309
siTip60
Tip60
Actin
C
0
8
16
24
32
40
48
56
64
72
Time P.I. (hrs)
Tip60
p53
EIB55K
E4orf6
Actin
D
0
30
60
90
120
CHX (min.)
Tip60
Actin
NI
Tip60
Actin
dl309
Control
dl309
150
100
50
0
%Tip60
0
30
60
90
120
Time (min)

Figure2


A
-E4
-E4orf6
-EIB55k
NI
dl309
dl366*
dl355
dl1520
Tip60
p53
Actin
B
HA-Tip60 - + + + +
EIB55K - - + - +
E4ORF6 - - - + +
GST - + + + +
HA
GST
Actin
C
NI
dl309
+dl309 MLN4924
+dl309 MG132
MLN4924 alone
MG132 alone
Tip60
Actin
D
HA-Tip60 - + + + +
EIB55K - - + + +
E4ORF6 - - + + +
GST - + + + +
MG132 - - - - +
MLN4924 - - - + -
HA
GST
Actin
E
Input
Input
IP:Flag
IP:Flag
IP:IgG
Input
IP:Flag
Input
IP:Flag
HA
EIB55K
E4orf6
1 2 3 4 5 6 7 8 9
F/H-Tip60 + + + + + + + + +
EIB55K + + + + + + + - -
E4orf6 + + + + + - - + +
MG132 - + - + + + + + +


mRNA level/actin
E4orf6
Pol
E4orf3
52/55K
Hexon
Fiber
0 24 48 72 96
X axis-hrs P.I.

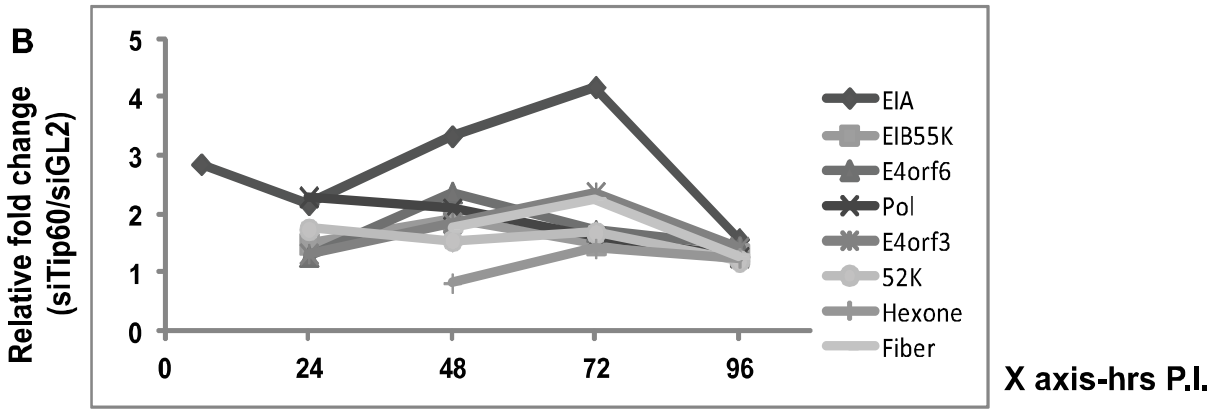

B
Relative fold change (siTip60/siGL2)
EIA
EIB55K
E4orf6
Pol
E4orf3
52K
Hexone
Fiber
0 24 48 72 96
X axis-hrs P.I.

Figure 4

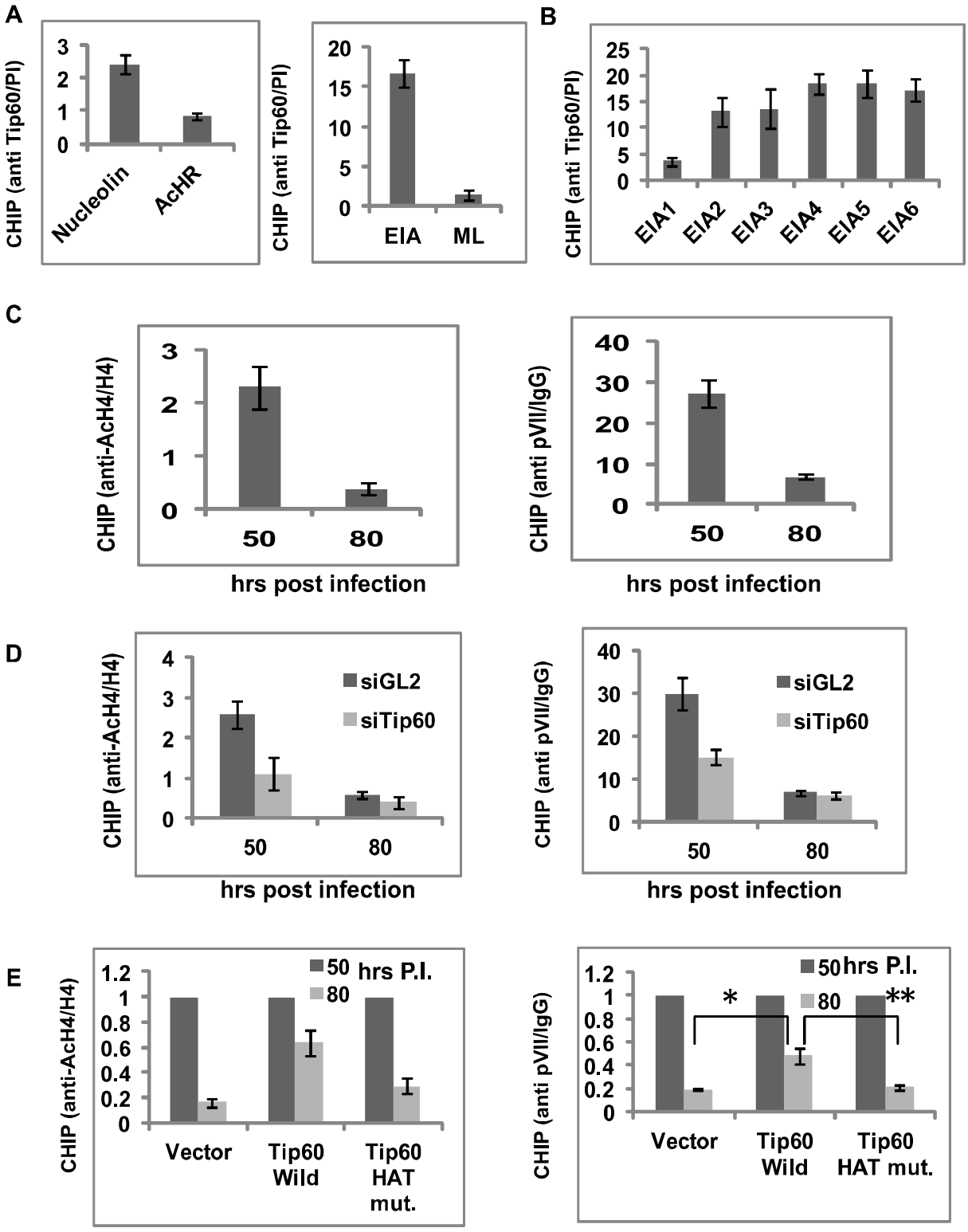

A
CHIP (anti Tip60/PI)
3
2
1
0
Nucleolin
AcHR
CHIP (anti Tip60/PI)
20
15
10
5
0
EIA
ML
B
CHIP (anti Tip60/PI)
25
20
15
10
5
0
EIA1
EIA2
EIA3
EIA4
EIA5
EIA6
C
CHIP (anti-AcH4/H4)
3
2
1
0
50
80
hrs post infection
CHIP (anti pVII/IgG)
40
30
20
10
0
50
80
hrs post infection
D
CHIP (anti-AcH4/H4)
4
3
2
1
0
siGL2
siTip60
50
80
hrs post infection
CHIP (anti pVII/IgG)
40
30
20
10
0
siGL2
siTip60
50
80
hrs post infection
E
CHIP (anti-AcH4/H4)
1.2
1
0.8
0.6
0.4
0.2
0
50 hrs P.I.
80
Vector
Tip60 Wild
Tip60 HAT mut.
CHIP (anti pVII/IgG)
1.2
1
0.8
0.6
0.4
0.2
0
50 hrs P.I.
80
*
**
Vector
Tip60 Wild
Tip60 HAT mut.

Figure 5

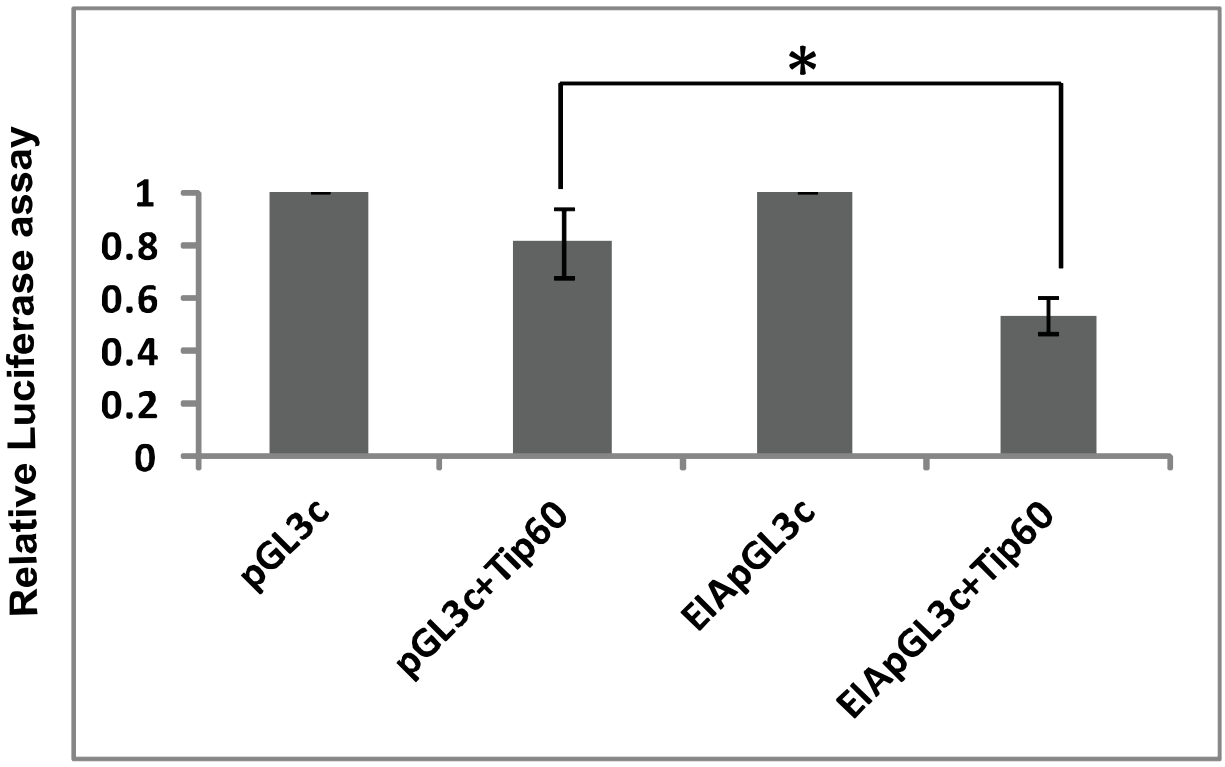

Relative Luciferase assay
1
0.8
0.6
0.4
0.2
0
*
pGL3c
pGL3c+Tip60
EIApGL3c
EIApGL3c+Tip60

Figure 6

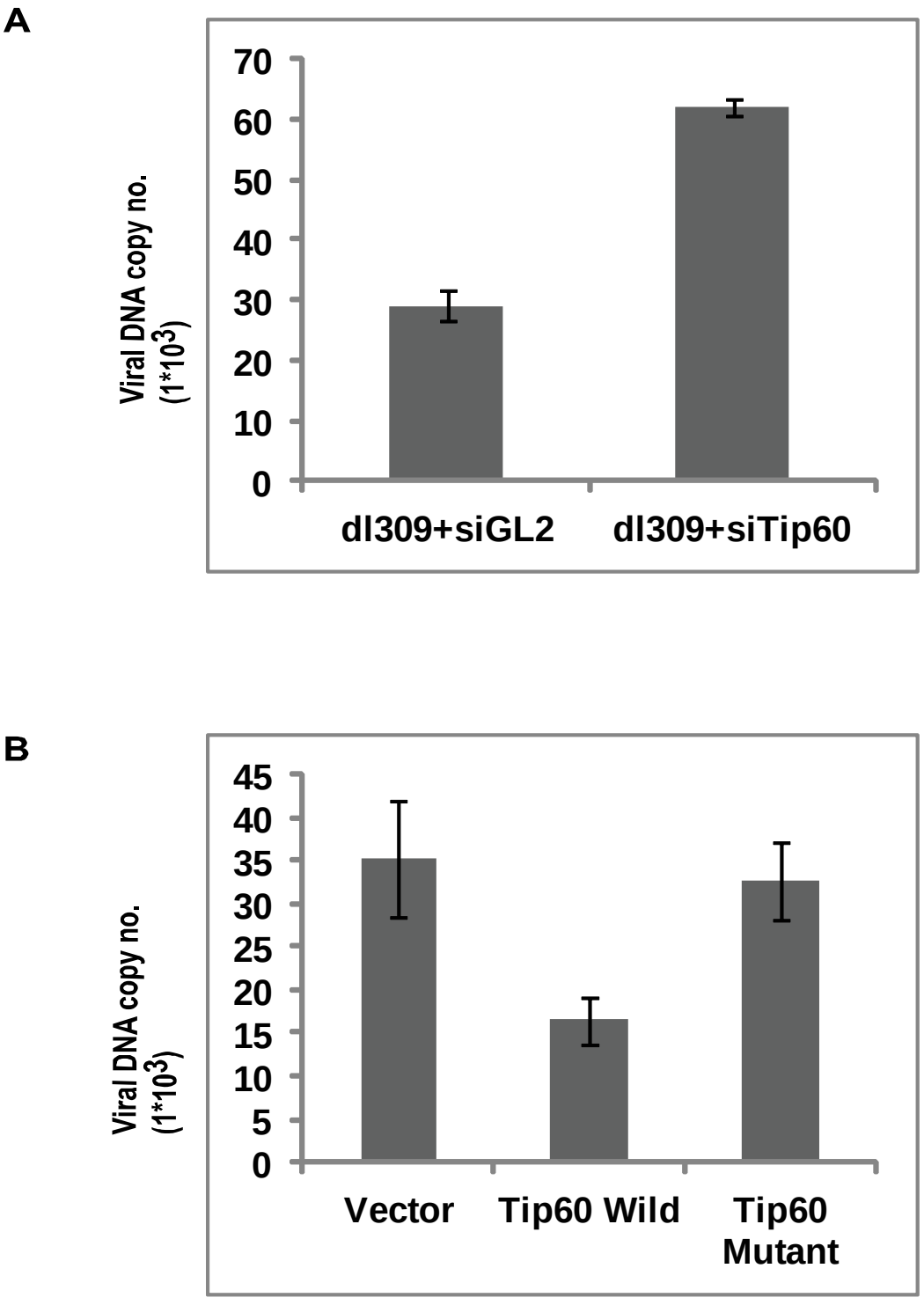

A
Viral DNA copy no. (1*10^3)
70
60
50
40
30
20
10
0
dl309+siGL2
dl309+siTip60
B
Viral DNA copy no. (1*10^3)
45
40
35
30
25
20
15
10
5
0
Vector
Tip60 Wild
Tip60 Mutant

Figure 7

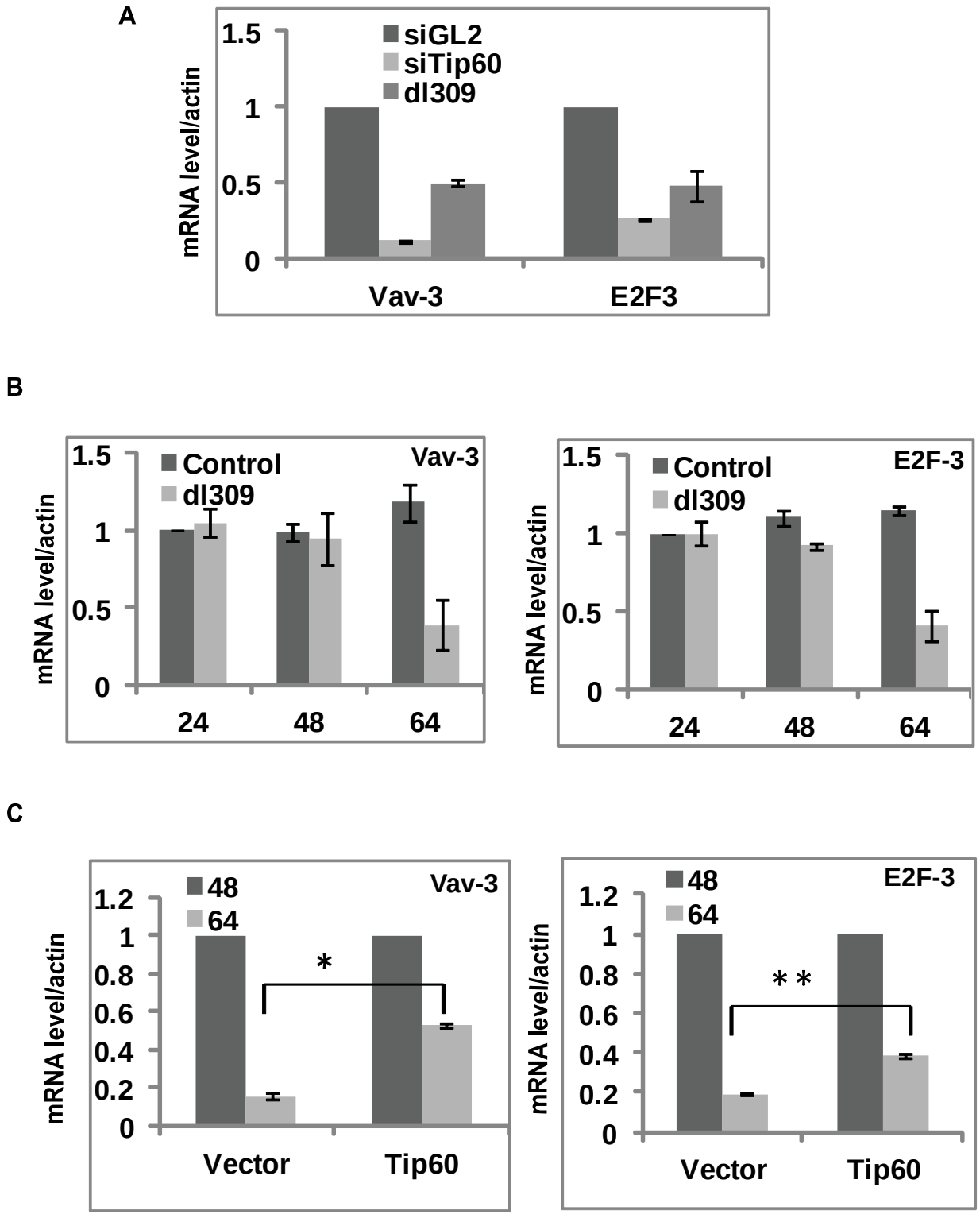
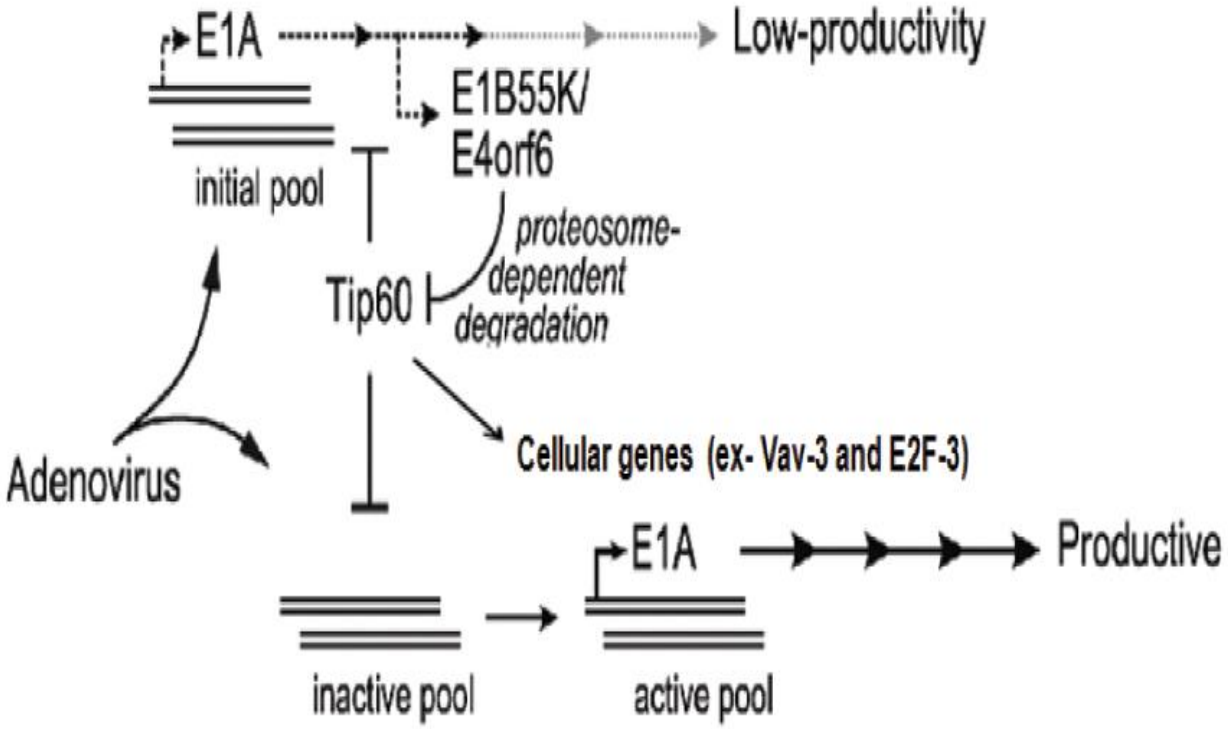
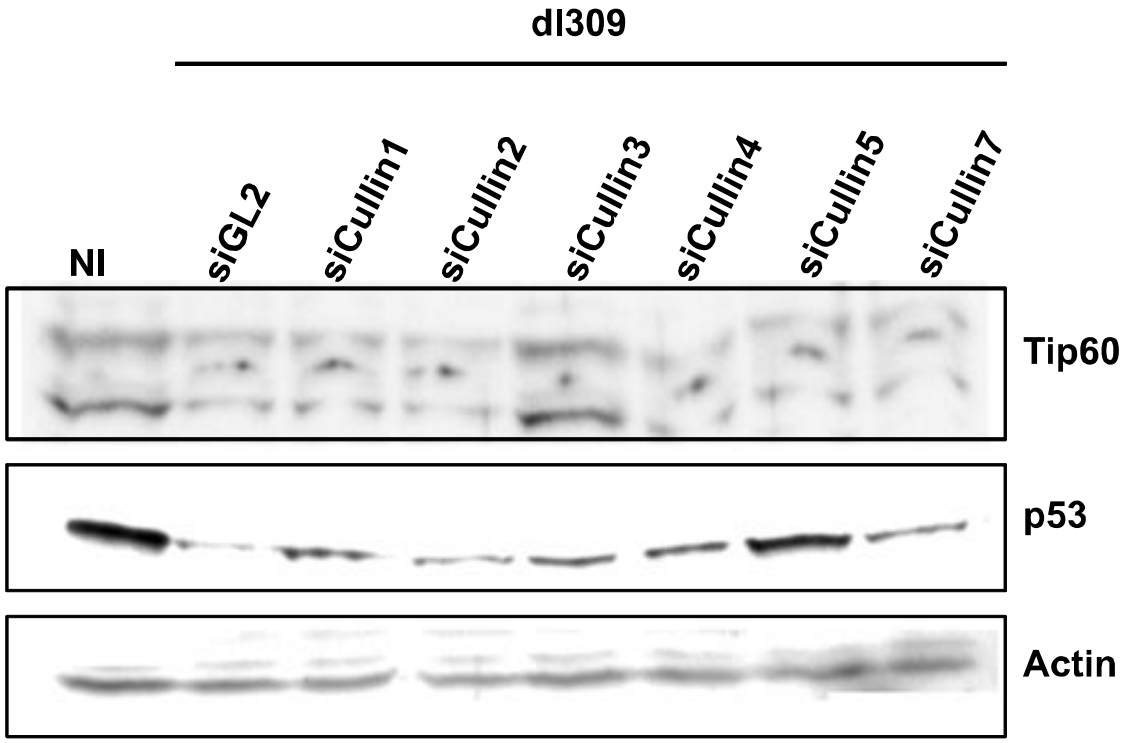

A
1.5
1
0.5
0
mRNA level/actin
siGL2
siTip60
dl309
Vav-3
E2F3
B
1.5
1
0.5
0
mRNA level/actin
Control
dl309
Vav-3
24
48
64
1.5
1
0.5
0
mRNA level/actin
Control
dl309
E2F-3
24
48
64
C
1.2
1
0.8
0.6
0.4
0.2
0
mRNA level/actin
48
64
Vav-3
*
Vector
Tip60
1.2
1
0.8
0.6
0.4
0.2
0
mRNA level/actin
48
64
E2F-3
**
Vector
Tip60

Figure 8

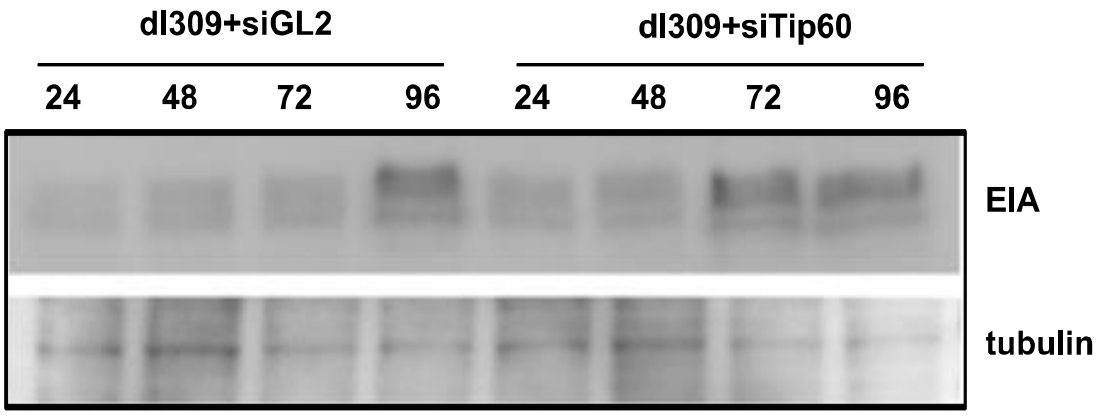

E1A
Low-productivity
E1B55K/
E4orf6
initial pool
proteosome-
dependent
degradation
Tip60
Adenovirus
Cellular genes (ex- Vav-3 and E2F-3)
E1A
Productive
inactive pool
active pool

FigureS1

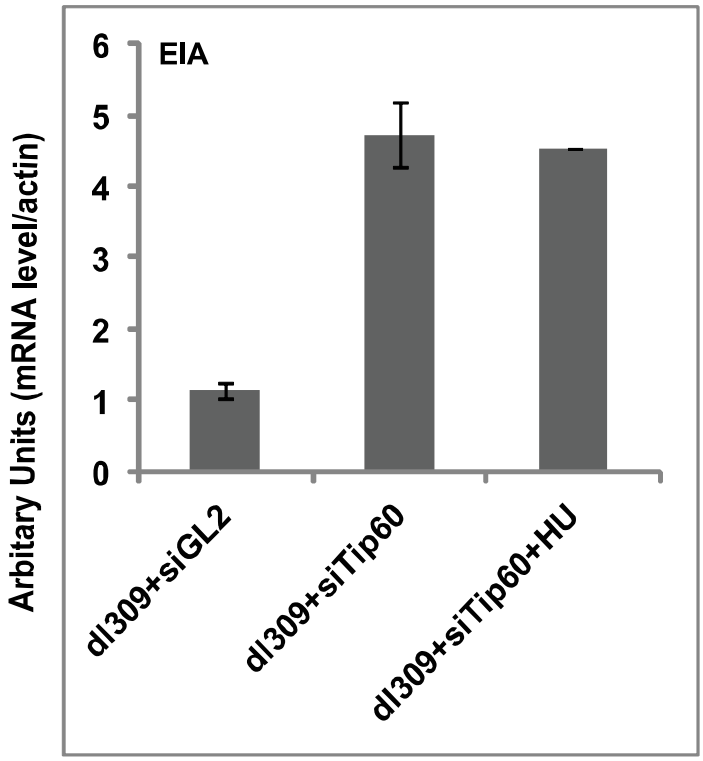

dl309
NI
siGL2
siCullin1
siCullin2
siCullin3
siCullin4
siCullin5
siCullin7
Tip60
p53
Actin

FigureS2

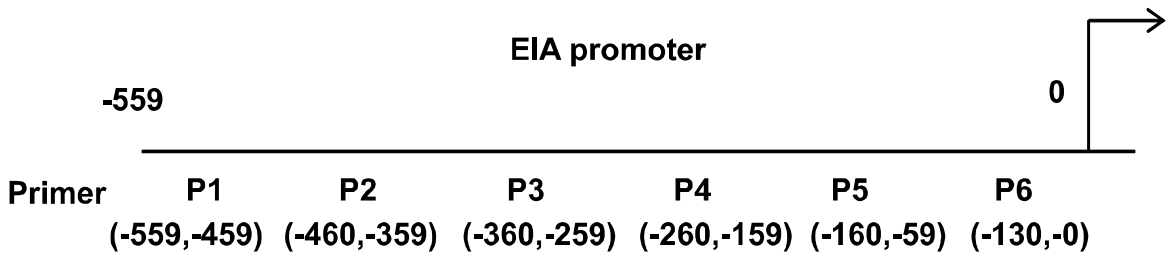
A


dl309+siGL2
dl309+siTip60
24
48
72
96
24
48
72
96
EIA
tubulin

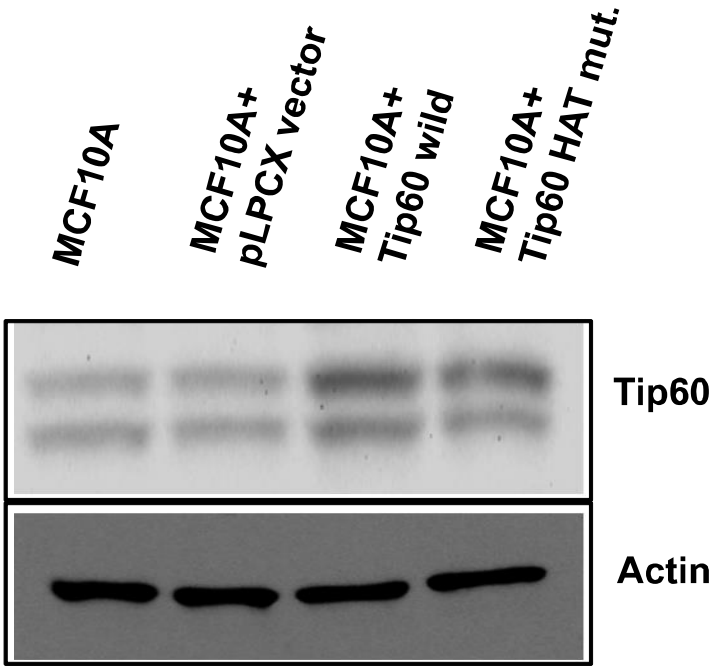
B


EIA
Arbitary Units (mRNA level/actin)
0
1
2
3
4
5
6
dl309+siGL2
dl309+siTip60
dl309+siTip60+HU

FigureS3


EIA promoter
-559
0
Primer
P1
P2
P3
P4
P5
P6
(-559,-459)
(-460,-359)
(-360,-259)
(-260,-159)
(-160,-59)
(-130,-0)

FigureS4


MCF10A
MCF10A+ pLPCX vector
MCF10A+ Tip60 wild
MCF10A+ Tip60 HAT mut.
Tip60
Actin