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Oncogene induced sensitization to chemotherapy-induced death requires induction as well as de-regulation of E2F1

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Abstract

The analysis of DNA tumor viruses has provided landmark insights into the molecular pathogenesis of cancer. A paradigm for this field has been the study of the adenoviral E1a protein which has led to identification of proteins such as p300, p400 and members of the retinoblastoma family. Through binding Rb family members, E1a causes deregulation of E2F proteins – an event common to most human cancers and a central pathway in which oncogenes, including E1a, sensitize cells to chemotherapy-induced programmed cell death. We report here, however, that E1a not only causes deregulation of E2F, but importantly that it also causes the post-transcriptional up-regulation of E2F1 protein levels. This effect is distinct from deregulation of E2F1, however, as mutants of E2F1 impaired in pRb binding are induced by E1a and E2F1 induction can also be observed in Rb-null cells. Analysis of E1a mutants selectively deficient in cellular protein binding revealed that induction of E2F1 is instead intrinsically linked to p400. Mutants unable to bind p400, despite being able to deregulate E2F1, do not increase E2F1 protein levels and they do not sensitize cells to apoptotic death. These mutants can, however, be complemented by either knockdown of p400, resulting in restoration of the ability to induce E2F1, or by over expression of E2F1, with both events re-enabling sensitization to chemotherapy-induced death. Due to the frequent deregulation of E2F1 in human cancer, these studies reveal potentially important insights into E2F1-mediated chemotherapeutic responses that may aid the development of novel targeted therapies for malignant disease.

Keywords

E2F1; E1a; apoptosis; protein-stability; p400

Introduction

At the cornerstone of our understanding of tumor development has been the mechanistic analysis of DNA tumor viruses, which has led to seminal discoveries such as the identification of p53 and members of the retinoblastoma family (1). Perhaps the most extensively studied amongst the oncoproteins from these viruses is the adenoviral E1a protein, which in combination with other oncogenes has potent transforming potential (2). Central to this activity was the discovery that E1a can inactivate the cellular Retinoblastoma protein, pRb (3). Inactivation of pRb also occurs in many human tumors in the absence of viral intervention and results in deregulation of the cellular E2F family which promotes cell-cycle progression (4, 5). Human cells have many checkpoints against malignant transformation and oncogenes often also sensitize cells to death in order to counter the potential replication of mutated cells. The deregulation of E2F1 is no exception which, while

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stimulating cell-cycle progression, also primes for cell death induction by many agents including chemotherapeutic drugs (5).

In addition to binding members of the Rb family, studies of E1a have also led to the identification of many other important cellular factors including p300/CBP, CtBP and p400 (6). p400 is the most recently described of these factors and has been reported to be critical for the transforming potential of E1a as well as for the ability of E1a to sensitize cells to death (7). We report here another layer of complexity in the way E1a sensitizes cells to death that has implications for the way in which cellular E2F1 can be modulated to enhance chemotherapy-induced tumor cell death.

Materials and Methods

Cell Culture, Transfections, Infections and RNAi

Primary Tert-immortalized retinal pigment epithelial (RPE) cells were obtained from ATCC. Authentication of these cells was undertaken by The European Collection of Cell Cultures within the last 6 months. MEF cells (wild-type, E2F1^{-/-} and Rb^{-/-} cells) were kindly provided by the laboratories of Nick Dyson and Tyler Jacks respectively. Genetic backgrounds of MEF lines were confirmed by PCR for each deleted allele in the last 6 months. Retroviral infections were undertaken as previously described (8). siRNAs were transfected using oligofectamine (Invitrogen) as previously described (9). siRNA sequences targeting *p400* were: GGAUACGCUGACAGAACAA (p400 siRNA #1), GCUUACACCAAUUGAAAAA (p400 siRNA #2) (Dharmacon). The scrambled non-silencing sequence was: TAGCGACTAAACACATCCAATT.

Plasmid, antibodies, immunoprecipitations and Western analyses

Expression vectors for E2F1 and truncations have been described (10). Retroviral constructs for full length and truncated forms of Ad5 E1a were gifts from Scott Lowe and Greg Hannon and have been previously described (11). pCMV-FLAG-p400 was kindly provided by David Livingston and has been previously described (12). pWZL-Blast-E1a was generated by cloning E1a sequences into the BamHI site of pWZL-Blast. pWZL-Blast-E1aER was a kind gift from Andy Phillips. Antibodies used were E2F1 (C20, Santa Cruz), E1a (BD Pharmingen), p400 (Bethyl Laboratories) and actin (clone 1A4, Sigma). C-terminal truncations of E2F1 were detected with KH20/KH95 (Upstate). Western analyses and immunoprecipitations were undertaken as previously described (13).

Adenoviruses

E2F1 sequences were cloned in to the EcoRV site of pShuttle-CMV (a kind gift from Bert Vogelstein) and subsequently recombined into pAdEasy-1. Control virus and adenovirus propagation were previously described (13).

Death Assays

After the treatments and times indicated, cells were observed visually for cell detachment and processed for flow cytometry as previously described (10). The percentage of cells with sub-G1 DNA content was taken as a measure of the percentage of apoptotic cells at that point in time (14).

qPCR

qPCR was undertaken as previously described (15). qPCR primers were from Qiagen: E2F1, Cat No. QT00016163; p400, Cat No. QT00079807. Primers for 18S have been previously described (16). Readings for E2F1 were normalized against 18S.

Results

E1a induces E2F1 protein levels in an RB-independent manner

Various chemotherapeutic drugs are known to induce E2F1 protein levels (Fig. 1A) (5). This induction, as shown here with the DNA damaging agent, adriamycin, is often a post-transcriptional event with no change in E2F1 mRNA levels (Fig. 1B). We significantly also observed that infection of primary, Tert-immortalized, human retinal pigment epithelial (RPE) cells with type 5 adenovirus 12S E1a also markedly induces E2F1 protein levels (Fig. 1C), without a concomitant increase in E2F1 mRNA (Fig. 1D).

E1a is known to regulate the activity of E2F1 by binding members of the retinoblastoma family and in particular by binding to the retinoblastoma protein itself, pRb (17-19). Since E1a-induced up-regulation of E2F1 has not previously been reported, we sought to determine if this new effect on E2F1 was also mediated through a similar mechanism involving pRb. Wild-type E2F1, as well as a mutant lacking the C-terminus of E2F1 which is deficient in Rb family binding, ($\Delta 374$), were therefore infected into E2F1^{-/-} MEFs which contain a transgene expressing E1aER (E1a fused to the hormone binding domain of the estrogen receptor) (20, 21). Treatment of these E1aER-expressing cells with the weak estrogen, tamoxifen, causes activation of E1a in this system. In the E2F1^{-/-} cells expressing the wild-type E2F1 transgene, E2F1 up-regulation was again observed following E1a activation confirming the result observed in RPE cells (Fig. 2A). E2F1- $\Delta 374$ levels were also increased following E1a activation, indicating that the mechanism of E2F1 up-regulation is distinct from E2F1 deregulation, as it does not simply occur by E1a binding to Rb family proteins and sequestering them away from E2F1 (Fig. 2A) (22).

Although pRb binding was not involved in the up-regulation of E2F1- $\Delta 374$ by E1a, it remained possible that the up-regulation of wild-type E2F1 may involve an interaction between E1a and pRB. To test this possibility, E1aER was infected into wild-type and Rb^{-/-} MEFs. In both cases, and in agreement with the result obtained with the E2F1- $\Delta 374$ mutant, treatment with tamoxifen caused a considerable increase in endogenous E2F1 protein levels (Fig. 2B). Taken together, therefore, these results show that E1a can up-regulate E2F1 protein levels and that this effect is independent of the binding of E1a to pRB (17-19).

Up-regulation of E2F1 and sensitization to chemotherapy-induced death requires the region of E1a which binds p400/TRRAP

To investigate further the mechanism by which E1a can increase E2F1 protein levels, we utilized a panel of previously described E1a mutants which lack regions required for binding different cellular proteins including p300/CBP, p400/TRRAP and CtBP (7, 11, 22). These mutants are therefore either selectively or collectively impaired in the engagement of these factors (Fig. 3A) (11). RPE cells were infected with these mutants and their expression assessed by Western blotting (Fig. 3B). The majority of the mutants, even the tr143 mutant which is expressed at relatively low levels, were able to induce E2F1 protein levels (Fig. 3B). The mutants $\Delta 2-24$, $\Delta 2-36$ and $\Delta 26-35$, however, were either unable or severely impaired in their ability to induce E2F1 (Fig. 3B). All of these E1a mutants retain an intact CR2 region and in line with this 2 of the mutants have previously been shown to bind pRb (22). As a result of pRB binding this will cause deregulation of E2F1, indicating once again that E2F1 induction is not simply a result of E2F1 transcriptional deregulation (7). Each of these mutants, however, share one common feature in that they are all unable to bind the cellular proteins p400/TRRAP (Fig. 3A). In contrast, all of the E1a mutants which are able to induce E2F1 retain the ability to bind p400/TRRAP (Fig. 3A) (7, 11, 22).

Since the ability of E1a to induce E2F1 protein levels required regions involved in p400/TRAP binding, we next tested if this correlated with the ability of E1a to sensitize to cell

death. The panel of E1a mutants were therefore infected into RPE cells and sensitivity to cell death assessed following treatment with adriamycin by observing the visual detachment of cells and the appearance of cells with sub-G1 DNA content by flow cytometry. This revealed, in an identical manner to what was observed for E2F1 induction, that those mutants lacking p400/TRRAP binding were refractory to death induction by adriamycin (Fig. 3C).

Knockdown of p400 enables E2F1 induction and sensitizes cells to chemotherapy-induced programmed cell death in cells expressing an E1a mutant lacking p400/TRRAP binding

Since only E1a mutants able to bind p400 were able to affect E2F1 protein levels, we next questioned if there could be a direct interaction between E2F1 and p400 in cells. In agreement with previous studies which have shown an interaction between E2F1 and the Tip60 acetyltransferase complex which has been shown to contain p400 (23, 24), immunoprecipitation of p400 with an antibody that does not cross-react with E2F1 (data not shown), could effectively bring down E2F1 in situations where both E2F1 and p400 were co-expressed in cells (Fig 4). In light of this result and the results with E1a deletion mutants, we next tested whether the inability of the E1a mutant Δ 26-35 to induce E2F1 protein levels could be connected to its inability to modulate p400. RPE cells and RPE cells expressing E1a- Δ 26-35 were therefore transfected with siRNAs targeting p400 or a scrambled siRNA as control. Both p400 targeted siRNAs caused a 70-80% reduction in p400 mRNA levels (Supplementary Fig. 1). Analysis of E2F1 by Western blotting revealed that knockdown of p400 either in the absence or presence of E1a- Δ 26-35 caused a marked increase in E2F1 protein levels (Fig. 5A).

We also questioned if the inability of E1a- Δ 26-35 to sensitize cells to chemotherapy-induced death could be rescued by knockdown of p400. RPE cells and RPE cells expressing E1a- Δ 26-35 were therefore subjected to p400 knockdown and subsequently treated with adriamycin. In RPE cells expressing E1a- Δ 26-35, despite increased cell death being observed in all cases due to the siRNA transfection, this revealed that knockdown of p400 caused a marked increase in the ability of these cells to undergo chemotherapy-induced programmed cell death as assessed by visible detachment of the cells from the dish and the appearance of cells with sub-G1 DNA content (Fig. 5B). This result was surprising as previous studies had shown that p400 knockdown could not rescue the ability of E1a- Δ 26-35 to sensitize cells to death (7). Although we are not clear about the reason for this discrepancy, this may simply be a reflection of the different cell types used in each study or the extent, or mode of action, of RNA interference used in the separate studies – acute (siRNA) in our study versus chronic (shRNA) used by Samuelson et al. (7).

In contrast to the effect we observed in RPE cells expressing E1a- Δ 26-35, no increase in cell death was observed, however, in RPE cells which had not been transduced with a virus expressing E1a- Δ 26-35 (Fig 5B), indicating that simple up-regulation of E2F1 as observed in Fig 5A is insufficient to sensitize cells to death and that other activities of E1a – presumably through the binding of Rb family members which leads to E2F1 transcriptional deregulation – are also required for effective sensitization to death (22).

Ectopic expression of E2F1 complements the lack of p400 binding by E1a mutants to sensitize cells to chemotherapy-induced programmed cell death

We next questioned whether the increased levels of E2F1 following p400 knockdown were potentially responsible for the increased sensitivity of cells to adriamycin. Mutant E1a expressing cells or control cells (which express an 'empty' pLPC retrovirus) were therefore infected with either an adenovirus expressing E2F1 or an adenovirus containing no transgene as control followed by treatment with adriamycin for 48h (Fig. 5C). In the

presence of mutant E1a, the ectopic expression of E2F1 caused a marked increase in the sensitivity of these cells to undergo adriamycin-induced death as assessed by visible detachment of the cells from the dish and the appearance of cells with sub-G1 DNA content (Fig. 5D). Expression of E2F1 alone, however, in the absence of adriamycin, did not cause cell death either in mutant E1a-expressing or control cells. Moreover, only a minimal increase in cell death was observed following adriamycin treatment of control cells (no E1a) that had been infected with E2F1 (Fig. 5D). These results, therefore, underscore the fact that it is both the increased expression, as well as the deregulation (22), of E2F1 by E1a that are required to sensitize cells to chemotherapy-induced programmed cell death.

Discussion

Taken together, these studies add a new and important layer of complexity that redefines our comprehension of the way the extensively studied E1a protein modulates the cellular Rb:E2F axis to sensitize cells to death. Since the actions of the E1a protein have been extensively studied, it is therefore vitally important to consider how these findings fit in with those previously provided by others.

In an almost parallel study, Tansey and co-workers have shown that E1a can cause the stabilization of c-Myc protein levels and that this stabilization is required for the ability of E1a to fully sensitize cells to chemotherapy-induced death (25, 26) (Fig. 6). The stabilization of c-Myc also involved the ability of E1a to bind p400, but this appears mechanistically distinct from the way in which E2F1 is stabilized. In the case of c-Myc, E1a was reported to facilitate the formation of a complex between c-Myc and p400 and this resulted in c-Myc protein stabilization (25). In the study we present here, however, it would be predicted by the fact that knockdown of p400 causes an increase in E2F1 protein levels (Fig 5), that a complex between p400 and E2F1 is not required for E2F1 stabilization to occur. Since we show that E2F1 can bind p400 (Fig 4), it could be predicted instead that E1a may sequester p400 away from E2F1 causing stabilization, although the way in which this ultimately leads to E2F1 stabilization is yet to be determined and we cannot completely discount the possibility that other proteins which bind in the E1a $\Delta 25-36$ region may also be involved (Fig. 6). It is tempting to speculate, however, that if E1a does take a component of the cellular p400 pool away from E2F1, it is then utilized by E1a to form c-Myc:p400 complexes resulting in sensitization to death by accumulation of both c-Myc and E2F1. In an attempt to address if this were the case, we firstly assessed if c-Myc levels were also increased in our cell systems following activation of E1aER in MEFs and RPE cells. In both cell types, although clear increases E2F1 protein levels could be detected, no increase in c-Myc was observed in either system (Supplementary Fig. 2). This clearly indicated therefore that the stabilization of c-Myc, and potentially also E2F1, by E1a are cell type and maybe context dependent.

Despite the fact that c-Myc was not up-regulated in our cell systems, the protein was clearly expressed and so it is still important to consider the numerous reports that have shown interplay between c-Myc and E2F1. On the one hand, E2F1 has been shown to increase expression of c-Myc and in a converse manner, c-Myc has been shown to modulate microRNA control of E2F1 expression and facilitate transactivation by E2F1 (27-30). In addition E2F1 and c-Myc have been shown to cooperate in cell death induction via p53 (31). Ultimately in Figure 6, we draw together the findings of our study with those of others on the effects of E1a on E2F1 and c-Myc. We also attempt to rationalize how E2F1 and c-Myc come together in the absence of E1a to ultimately regulate sensitivity to death. Where questions remain is also highlighted and these undoubtedly represent areas worthy of further investigation (Fig 6).

Although many questions still remain, since deregulation of E2F1 is a facet of multiple human cancers and since many chemotherapeutic drugs independently cause up-regulation of E2F1 protein levels, the potential implications of these findings may well be more widespread than studies focused on E1a. Subsequent studies in this regard, particularly with respect to the use of differing concentrations of adriamycin and additional chemotherapeutic drugs, will therefore hopefully lead to a fuller understanding of the generality and potential consequences of this important observation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Howley PM, Livingston DM. Small DNA tumor viruses: large contributors to biomedical sciences. *Virology*. 2009; 384:256–9. [PubMed: 19136134]
2. Ruley HE. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature*. 1983; 304:602–6. [PubMed: 6308473]
3. Whyte P, Buchkovich KJ, Horowitz JM, et al. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature*. 1988; 334:124–9. [PubMed: 2968522]
4. Trimarchi JM, Lees JA. Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol*. 2002; 3:11–20. [PubMed: 11823794]
5. Bell LA, Ryan KM. Life and death decisions by E2F-1. *Cell Death Differ*. 2004; 11:137–42. [PubMed: 14526389]
6. Frisch SM, Mymryk JS. Adenovirus-5 E1A: paradox and paradigm. *Nat Rev Mol Cell Biol*. 2002; 3:441–52. [PubMed: 12042766]
7. Samuelson AV, Narita M, Chan HM, et al. p400 is required for E1A to promote apoptosis. *J Biol Chem*. 2005; 280:21915–23. [PubMed: 15741165]
8. O'Prey J, Wilkinson S, Ryan KM. Tumor antigen LRRC15 impedes adenoviral infection: implications for virus-based cancer therapy. *J Virol*. 2008; 82:5933–9. [PubMed: 18385238]
9. Crighton D, Wilkinson S, O'Prey J, et al. DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. *Cell*. 2006; 126:121–34. [PubMed: 16839881]
10. Bell LA, O'Prey J, Ryan KM. DNA-binding independent cell death from a minimal proapoptotic region of E2F-1. *Oncogene*. 2006; 25:5656–63. [PubMed: 16652153]
11. Seger YR, Garcia-Cao M, Piccinin S, et al. Transformation of normal human cells in the absence of telomerase activation. *Cancer Cell*. 2002; 2:401–13. [PubMed: 12450795]
12. Fuchs M, Gerber J, Drapkin R, et al. The p400 complex is an essential E1A transformation target. *Cell*. 2001; 106:297–307. [PubMed: 11509179]
13. Bell HS, Dufes C, O'Prey J, et al. A p53-derived apoptotic peptide derepresses p73 to cause tumor regression in vivo. *J Clin Invest*. 2007; 117:1008–18. [PubMed: 17347683]
14. Pellicciari C, Manfredi AA, Bottone MG, Schaack V, Barni S. A single-step staining procedure for the detection and sorting of unfixed apoptotic thymocytes. *Eur J Histochem*. 1993; 37:381–90. [PubMed: 7510545]
15. O'Prey J, Skommer J, Wilkinson S, Ryan KM. Analysis of DRAM-related proteins reveals evolutionarily conserved and divergent roles in the control of autophagy. *Cell Cycle*. 2009; 8:2260–5. [PubMed: 19556885]

16. Crichton D, O'Prey J, Bell HS, Ryan KM. p73 regulates DRAM-independent autophagy that does not contribute to programmed cell death. *Cell Death Differ.* 2007; 14:1071–9. [PubMed: 17304243]
17. Bagchi S, Raychaudhuri P, Nevins JR. Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A trans- activation. *Cell.* 1990; 62:659–69. [PubMed: 2143697]
18. Bandara LR, La Thangue NB. Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. *Nature.* 1991; 351:494–7. [PubMed: 1710781]
19. Chellappan SP, Hiebert S, Mudryj M, Horowitz JM, Nevins JR. The E2F transcription factor is a cellular target for the RB protein. *Cell.* 1991; 65:1053–61. [PubMed: 1828392]
20. Helin K, Harlow E. Heterodimerization of the transcription factors E2F-1 and DP-1 is required for binding to the adenovirus E4 (ORF6/7) protein. *J Virol.* 1994; 68:5027–35. [PubMed: 8035503]
21. Spitkovsky D, Steiner P, Lukas J, et al. Modulation of cyclin gene expression by adenovirus E1A in a cell line with E1A-dependent conditional proliferation. *J Virol.* 1994; 68:2206–14. [PubMed: 8139005]
22. Samuelson AV, Lowe SW. Selective induction of p53 and chemosensitivity in RB-deficient cells by E1A mutants unable to bind the RB-related proteins. *Proc Natl Acad Sci U S A.* 1997; 94:12094–9. [PubMed: 9342368]
23. Taubert S, Gorrini C, Frank SR, et al. E2F-dependent histone acetylation and recruitment of the Tip60 acetyltransferase complex to chromatin in late G1. *Mol Cell Biol.* 2004; 24:4546–56. [PubMed: 15121871]
24. Ikura T, Ogryzko VV, Grigoriev M, et al. Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. *Cell.* 2000; 102:463–73. [PubMed: 10966108]
25. Tworkowski KA, Chakraborty AA, Samuelson AV, et al. Adenovirus E1A targets p400 to induce the cellular oncoprotein Myc. *Proc Natl Acad Sci U S A.* 2008; 105:6103–8. [PubMed: 18413597]
26. Chakraborty AA, Tansey WP. Adenoviral E1A function through Myc. *Cancer Res.* 2009; 69:6–9. [PubMed: 19117980]
27. Leung JY, Ehmann GL, Giangrande PH, Nevins JR. A role for Myc in facilitating transcription activation by E2F1. *Oncogene.* 2008; 27:4172–9. [PubMed: 18345030]
28. Elliott MJ, Dong YB, Yang H, McMasters KM. E2F-1 up-regulates c-Myc and p14(ARF) and induces apoptosis in colon cancer cells. *Clin Cancer Res.* 2001; 7:3590–7. [PubMed: 11705881]
29. Wong KK, Zou X, Merrell KT, et al. v-Abl activates c-myc transcription through the E2F site. *Mol Cell Biol.* 1995; 15:6535–44. [PubMed: 8524218]
30. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature.* 2005; 435:839–43. [PubMed: 15944709]
31. Hong S, Pusapati RV, Powers JT, Johnson DG. Oncogenes and the DNA damage response: Myc and E2F1 engage the ATM signaling pathway to activate p53 and induce apoptosis. *Cell Cycle.* 2006; 5:801–3. [PubMed: 16582589]

Figure 1. DNA damage and E1a cause post-transcriptional induction of E2F1
 (A+B) RPE cells were treated with 2 μ g/ml adriamycin (Adr) for the indicated times. Protein and RNA were prepared and analysed respectively by Western blotting for E2F1 and actin (A) and by qPCR for E2F1 mRNA (B). (C+D) RPE cells were infected with retroviruses expressing Ad5-12S-E1a, E2F1 or empty retroviral vector (pLPC) as control. Protein and RNA were prepared and analysed respectively by Western blotting for E2F1, E1a and actin (C) and by qPCR for levels of E2F1 mRNA (D).

Figure 2. Induction of E2F1 by E1a does not involve pRb

(A) E2F^{-/-} MEFs were infected with a retrovirus expressing E1a-ER. Cells were then super-infected with retroviruses expressing wild-type human E2F1 and the E2F1 mutant Δ 374. Where indicated, cells were treated with tamoxifen (Tam) for 24h. Protein lysates were prepared from cells and analysed by Western blotting for E2F1 and actin. (B) Wild-type and Rb^{-/-} MEFs were infected using a retrovirus expressing E1aER. Where indicated, cells were treated with Tam for 24h. Protein lysates were prepared from cells and analysed by Western blotting for E2F1 and actin.

Figure 3. E1a mutants lacking p400 binding cannot induce E2F1 and are impaired in sensitization to chemotherapy-induced death

(A) E1A mutants which selectively lack the ability to bind cellular proteins were retrovirally transduced into RPE cells. The mutants lack amino acids as defined. The mutant R2G comprises full-length E1a with the 2nd arginine mutated to glycine. The mutant tr143 consists of the first 143 amino acids of E1a. (B) Protein lysates were prepared from cells and analysed by Western blotting for E1a, E2F1 and actin. (C) Cells containing the E1a mutants, cells containing the empty retroviral vector (pLPC) and uninfected RPE cells were treated with 0.5µg/ml adriamycin for 48h. Cell populations were then analysed for cell death by flow cytometry.

Figure 4. E2F1 interacts with p400

Cells were transiently transfected with the indicated plasmids. Cell lysates were generated and immunoprecipitated with an antibody against p400. Lysates were then assayed by Western analyses for the presence of p400, E1a and E2F1. Input lysates were also assayed by Western blotting to confirm equal expression in different samples of E2F1, E1a and actin.

Figure 5. The activity of E1a mutants deficient in p400 binding can be complemented by p400 knockdown or ectopic expression of E2F1

(A) RPE cells and RPE cells expressing the E1a mutant $\Delta 26-35$ were transfected with 2 different siRNAs targeting p400 or a scrambled siRNA (Scr) as control. Protein lysates were prepared from cells and analysed by Western blotting for E2F1 and actin. (B) RPE cells and RPE cells expressing $\Delta 26-35$ and which had been subjected to either p400 or control siRNA transfection were treated with 0.1 $\mu\text{g/ml}$ adriamycin (Adr) for 48h and analysed for cell death by flow cytometry. (C+D) RPE cells expressing $\Delta 2-36$ or empty viral vector as control (Cont.) were infected with an adenovirus lacking E1 sequences which expresses wild-type E2F1 or empty adenoviral vector as control (also lacking E1 sequences). Where indicated cells were treated with 0.5 $\mu\text{g/ml}$ adriamycin (Adr) for 48h. Cell lysates were prepared and analysed by Western blotting for E2F1, E1a and actin (C). Cell populations were also analysed for cell death by flow cytometry (D).

Figure 6. A model summarizing the reported effects of E1a on c-Myc, E2F1 and p400

Previous studies have shown, and we report here, that E2F1 can bind pRb and p400. Numerous previous studies have also shown that E1a can bind pRb and p400. Binding of pRb by p400 results in the transcriptional deregulation of E2F1. From the studies we report here, we propose that E1a also sequesters p400 away from E2F1 and causes stabilization of E2F1 protein levels. Both up-regulation and transcriptional deregulation of E2F1 are required for full sensitization to chemotherapy-induced death. Previous studies have also reported that E1a promotes an interaction between p400 and c-Myc and this causes stabilization of c-Myc protein levels. Stabilization of c-Myc is also required for complete sensitization to death. Various studies have reported cross-talk between E2F1 and c-Myc.

Questions still remain at this point and at other points indicated by '?' in the schematic. These questions are discussed further in the main body of the manuscript.