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Heterochromatin silencing at p53 target genes by a small viral protein

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Abstract

The transcription factor p53 guards against tumor and virus replication and is inactivated in almost all cancers. p53 activated transcription of target genes is thought to be synonymous with the stabilization of p53 in response to oncogenes and DNA damage. During adenovirus replication, the degradation of p53 by E1B-55k is considered essential for p53 inactivation, and is the basis for p53 selective viral cancer therapies. Here we reveal a dominant epigenetic mechanism that silences p53-activated transcription, irrespective of p53 phosphorylation and stabilization. We show that another adenoviral protein, E4-ORF3, inactivates p53 independently of E1B-55k by forming a nuclear structure that induces *de novo* H3K9me3 heterochromatin formation at p53 target promoters, preventing p53 DNA-binding. This suppressive nuclear web is highly selective in silencing p53 promoters and operates in the backdrop of global transcriptional changes that drive oncogenic replication. These findings are important for understanding how high levels of wild-type p53 might also be inactivated in cancer as well as the mechanisms that induce aberrant epigenetic silencing of tumor suppressor loci. Our study changes the longstanding definition of how p53 is inactivated in adenovirus infection and provides key insights that could enable the development of true p53 selective oncolytic viral therapies.

Tumor mutations and DNA virus proteins converge in inactivating p53 1, which was initially discovered as a cellular target of SV40 Large T 2,3. However, despite 30 years of research, the critical factors that determine p53 activated transcription are still not fully understood 4,5. p53 is expressed constitutively in normal cells where its activity is limited

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Author Contributions

C.S. and F.E.E. contributed equally to this work. C.S. performed the p53 activation and virus studies, including immunoblotting, RT-QPCR and microarray experiments. F.E.E. performed all chromatin immunoprecipitation and immunofluoresence studies. K.C.E. performed the luciferase assays, E4-ORF3 sufficiency and complementation, and assisted C.S with viral mutant studies. C.C.O.S. analyzed the array data and wrote the paper with contributions from all authors. C.C.O.S. was responsible for the overall conceptual design and supervision of the studies.

Full methods are available online. Microarray data are deposited in NCBI's Gene Expression Omnibus (GSE20607). Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests.

Supplementary Information

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by p53 protein degradation 6. p53 activation is triggered in response to oncogenes and DNA damage, which stabilize p53 7–9. This has led to the general belief that the induction of p53 levels and phosphorylation 10 is synonymous with p53 activated transcription. As such, the induction of p53 levels is a standard read-out for p53 activation and rationale for several cancer therapies, including irradiation and genotoxic drugs 11, MDM2 antagonists 12, and the E1B-55k deleted oncolytic adenoviral therapy, ONYX-015 13.

The adenoviral protein, E1B-55k, binds to the p53 transactivation domain and is sufficient to inactivate p53 in cellular transformation 7,14. In infection, E1B-55k forms a complex with another adenoviral protein, E4-ORF6, which recruits a cellular ubiquitin ligase that targets p53 for degradation 15,16. The degradation of p53 by E1B-55k is thought to be the critical event that inactivates p53 for virus replication 17. An E1B-55k deleted virus18, *dl*1520/ ONYX-015, induces high p53 levels, which was expected to limit viral replication in normal cells but not p53 mutant tumor cells 13. On this basis, ONYX-015 13 was tested in patients as a p53 tumor selective oncolytic viral therapy 19,20 and is now approved in several countries (known as Oncorine). However, the loss of E1B-55k functions in viral RNA export, rather than p53 inactivation, is the major determinant of ΔE1B-55k tumor selectivity 21,22. Contrary to expectations, although p53 accumulates to high levels in the nucleus of ΔE1B-55k (Δ55k) infected human primary small airway epithelial cells (SAECs), the physiological target cells for adenovirus infection, p53 transcriptional targets, such as, *p21*, *MDM2*, *Cyclin G*, *14-3-3σ*, *PERP*, *PIG3* and *GADD45* are not induced (Fig 1a and ref 21). The failure of p53 stabilization to activate transcriptional targets is not a tissue specific effect and occurs in multiple primary cell types and tumor cell-lines (Supp. Fig 1–3), including U2OS tumor cells where p53 targets are suppressed to a similar extent as that in cell-lines with p53 mutations. This reveals a fundamental gap in our understanding of not only adenovirus biology but also p53 activation.

p53 stabilization without activity

Cellular and viral oncogenes, such as Ras and Adenovirus E1A, trigger p53 activation by inducing the expression of ARF 9, which inhibits MDM2 mediated p53 degradation. ARF is lost in 58% of cancers9, which had previously been invoked as the critical factor that prevents p53 activation in ΔE1B-55k infected tumor cells 23. Using a U2OS stable cell-line (p53 wild-type, ARF negative) in which ARF expression is induced by isopropyl-β-D-thiogalactopyranoside (IPTG), we show that ARF stabilizes p53 and activates p21 transcription in mock infection. Nevertheless, although ARF expression increases basal p53 activity, the induction of p21 is repressed in both wild-type (wt) and ΔE1B-55k infected cells (Fig 1b and Supp. Fig 4). Furthermore, endogenous ARF induction also fails to activate p53 targets inΔE1B-55k infected SAECs (Fig 1a). Thus, p53 is inactivated, irrespective of E1B-55k and ARF expression in adenovirus infected cells.

DNA damage signals also play a critical role in activating p53, triggering p53 phosphorylation and protein stabilization 8,24. In clinical trials, ΔE1B-55k (ONYX-015), was used in combination with genotoxic chemotherapies, such as 5-fluorouracil (5-FU) 19,20. We reasoned that the induction of p53 levels alone may not be sufficient to activate p53 in infected cells, and that DNA damage is also required. However, 5-FU fails to activate

p53 in ΔE1B-55k infected U2OS cells (Supp. Fig 5). The DNA damage checkpoint is deregulated in many tumor cells. Therefore, we also analyzed ΔE1B-55k infected SAECs and show that p53 transcriptional targets cannot be activated by γ irradiation (Fig 1c and Supp. Fig 6), UV irradiation (Supp. Fig 7) or doxorubicin (dox, Fig 1e).

The activation of p53 in response to DNA damage is mediated via kinases, such as ATM, ATR, DNA-PKcs, CHK1 and CHK2, which phosphorylate p53 8 at key residues, stabilizing p53 and potentiating p53 DNA binding 24. A possible explanation for the failure of DNA damage to activate high p53 levels in ΔE1B-55k infected cells is that p53 phosphorylation is inhibited by viral infection. However, even without the introduction of exogenous genotoxic stress, p53 is already highly phosphorylated at multiple sites targeted by DNA damage kinases in ΔE1B-55k infected SAECs (Fig 1d). Thus, although oncogenes and DNA damage trigger p53 stabilization and phosphorylation inΔE1B-55k infected cells, p53 fails to activate the transcription of downstream effectors.

We next examined if in the absence of E1B-55k, MDM2 binds and inactivates p53 in adenovirus infected cells. Nutlin is a small molecule antagonist that inhibits MDM2-p53 binding 12. In contrast to mock, nutlin fails to stabilize p53 further or induce p21 in ΔE1B-55k infected SAECs (Fig 1e). The histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), induces the expression of p21 independently of p53 stabilization or phosphorylation (Fig 1e). However, in ΔE1B-55k infected cells, TSA fails to induce p21. We conclude that p53 transcriptional targets are dominantly suppressed in adenovirus infected cells, irrespective of E1B-55k, and cannot be activated in response to radiation, genotoxic drugs, ARF, MDM2 antagonists or HDAC inhibitors.

E4-ORF3 inactivates p53 independently of E1B-55k

Our data strongly suggest that there is a previously undiscovered adenoviral protein that inactivates p53 independently of p53 degradation. To test this, we screened for p53 activation in primary cells infected with adenoviruses that have compound mutations in E1B-55k and other early viral genes (Supp. Fig 8). In addition to deleting E1B-55k, the loss of either E1A-13s or E4-ORF3 is required to activate p53 in infected cells (Fig 2a). This is surprising, especially since E1A is a potent oncogene that triggers p53 activation in cellular transformation 7,14. In adenovirus infection, the E1A-13s splice form is required for the transactivation of other viral genes 17, including E4-ORF3 (Fig 2a). Consistent with this, we show that in contrast to GFP, the ectopic expression of E4-ORF3 rescues p53 inactivation in both ΔE1B-55k/ΔE4-ORF3 and ΔE1B-55k/ΔE1A-13s infected cells (Fig 2b). The slight reduction of p21 by Ad-GFP inΔE1B-55k/ΔE4-ORF3 co-infection is due to the partial activation of E4-ORF3 transcription (*in trans*) by E1A-13s, which does not occur inΔE1B-55k/ΔE1A-13s co-infection (Supp. Fig 9). Hence, E1A-13s induces the expression of E4-ORF3 which then inactivates p53 *via* an E1B-55k independent mechanism. Moreover, the expression of E4-ORF3 alone is also sufficient to inhibit p53 activation (Supp. Fig 10). These data reveal E4-ORF3 as a novel adenoviral protein that inactivates the p53 tumor suppressor pathway.

The proposed p53 tumor selectivity of the ΔE1B-55k oncolytic therapy, ONYX-015, is based on p53 stabilization being the sole critical event that determines p53 activated transcription. Although there is some basal p53 activity in ΔE1B-55k infected cells compared to wild-type virus, the additional deletion of E4-ORF3 is necessary for p53 to activate downstream effectors over the course of infection (Fig 2c-d). In contrast to p53 transcriptional targets, the mRNA levels of p53 and the housekeeping gene, *GUSB,* are not impacted by E4-ORF3 (Supp. Fig 11). p53 stabilization is required to activate p53 transcriptional targets, and does not occur in ΔE4-ORF3 infection where p53 is degraded by E1B-55k/E4-ORF6. Furthermore, using a p53 inducible stable cell-line (H1299-D1, Supp. Fig 12), we show that the induction of p21 and MDM2 in ΔE1B-55k/ΔE4-ORF3 infection is p53-dependent. Thus, the deletion of both E1B-55k and E4-ORF3 is necessary to activate p53 in adenovirus infection. We conclude that E4-ORF3 has a critical and novel role in inactivating p53 independently of E1B-55k and p53 degradation.

E4-ORF3 prevents p53-DNA binding at chromatin

DNA tumor virus proteins, such as E1B-55k, SV40 LT and HPV E6 inactivate p53 *via* direct high affinity protein-protein interactions 1. However, contrary to this established paradigm, E4-ORF3 does not co-localize with p53 (Supp. Fig 13) or co-immunoprecipitate with p53 (data not shown). This suggests that E4-ORF3 inactivates p53 *via* a non-canonical mechanism.

The induction of p53 levels and phosphorylation induces p53 conformational changes that drive sequence-specific DNA binding and the recruitment of transcription co-factors 25. A p53 DNA binding domain that is competent to bind to DNA can be distinguished by immunoprecipitation with PAb 1620 *versus* PAb 240 26. p53 is immunoprecipitated selectively by PAb 1620 in bothΔE1B-55k and ΔE1B-55k/ΔE4-ORF3 infected cells (Supp. Fig 14), demonstrating that the p53 DNA binding domain is in a protein conformation that should be capable of binding to DNA 26 in both cases. To functionally determine if E4-ORF3 prevents p53 DNA binding, we transfected U2OS cells with a p53 luciferase plasmid (p53-luc), where p53 binding to consensus DNA sequences activates luciferase transcription 27. A control pGL3-luciferase reporter (non-p53 promoter) is activated to similar levels in all viral infections (Supp. Fig 15). In wild-type virus infected cells, p53 activated transcription of luciferase is inhibited after 24 hours (Fig 3a), which is expected due to p53 degradation. In contrast, p53-luciferase is activated in both ΔE1B-55k and ΔE1B-55k/ΔE4-ORF3 infection (Fig 3a). The induction of luciferase requires p53 DNA binding, since a mutated p53 response element (p53-mutant) abolishes luciferase activity. These experiments demonstrate that E4-ORF3 does not compete with p53 for binding to consensus DNA target sequences or prevent p53 transcriptional activation of promoters in ectopic reporter plasmids.

The ability of E4-ORF3 to prevent p53 activated transcription of endogenous targets but not ectopic p53-luciferase plasmids is at first difficult to reconcile. Plasmid DNA is not subject to the same architectural and packing constraints as DNA in cellular chromatin. Therefore, we performed p53 chromatin immunoprecipitations (ChIPs) to determine if E4-ORF3 specifically prevents p53 DNA binding in the context of cellular chromatin. p53 binding to

target sites in the *p21* (5′ and 3′ site) and *MDM2* promoters 25 is induced upon doxorubicin treatment and ΔE1B-55k/ΔE4-ORF3 infection, where it activates the transcription of *p21* and *MDM2* RNAs (Fig 3b-c). In contrast, although p53 is induced to similar levels, E4-ORF3 prevents p53 DNA binding to the *p21* and *MDM2* promoters in ΔE1B-55k infected cells (Fig 3b–c and Supp. Fig 16). Thus, E4-ORF3 inactivates p53 by preventing p53 binding to DNA target sites specifically in the context of cellular chromatin.

Repressive histone methylation silences p53 targets

We reasoned that p53 DNA binding depends not only on the protein conformation of p53 but also the accessibility of target promoters in the cellular genome. We hypothesized that E4-ORF3 could inactivate p53 by inducing heterochromatin at endogenous target promoters, preventing the access of p53 to DNA. Heterochromatin compaction is specified by the loss of histone acetylation and induction of repressive histone methylation 28. TSA fails to induce p21 in ΔE1B-55k infected SAECs (Fig 1e) suggesting that E4-ORF3 inactivates p53 targets *via* a mechanism that is dominant to the inhibition of histone deacetylation. In cancer, the aberrant epigenetic silencing of tumor suppressor genes, such as $p16^{INK4a}$, is initiated by the methylation of histone H3 at lysine 9 (H3K9) 29. p53 localization is indistinguishable in ΔE1B-55k and ΔE1B-55k/ΔE4-ORF3 infected cells (Fig 3d). However, in ΔE1B-55k infected cells, where p53 is inactive, dense regions of H3K9me3 repressive heterochromatin are induced at the periphery of the nucleus (Fig 3d and Supp. Fig 17). Of the four known methyltransferases that catalyze H3K9 trimethylation, we show that SUV39H1 and SUV39H2 (which share 59% sequence identity and have redundant functions) 30,31, but not SETDB1 32 or G9a 33, are specifically associated with the formation of *de novo* H3K9me3 heterochromatin domains in ΔE1B-55k infected nuclei (Fig 3e). The formation of these domains requires E4-ORF3 and does not occur in either mock or ΔE1B-55k/ΔE4-ORF3 infected cells (Supp. Fig 18–21).

These data demonstrate that E4-ORF3 induces novel H3K9me3 heterochromatin, which could deny p53 access to endogenous target promoters. To test this, we performed p53 and H3K9me3 ChIPs. The induction of repressive heterochromatin by E4-ORF3 is not associated with a global upregulation of either total histone H3 or H3K9me3, which are at similar levels in all infections (Fig 4a). In ΔE1B-55k/ΔE4-ORF3 infected cells, p53 binding is induced at *p21* and *MDM2* promoter sites (consistent with Fig 3c) while H3K9me3 is at a similar level to an IgG negative control (Fig 4a and Supp. Fig 22). In contrast, in ΔE1B-55k infected cells, H3K9me3 is enriched at the *p21* and *MDM2* promoters where p53 binding is prevented. H3K9me3 is also induced at the -5kb region of the *p21* promoter and is not restricted to p53 binding sites (Supp. Fig 22). Thus, in cells expressing E4-ORF3, there is an inverse correlation between p53 and H3K9me3 at p53 regulated promoters. The same conclusions were reached for additional p53 targets, including *GADD45A, FAS*, *PUMA* and *PIG3* (Supp. Fig 22–24). In contrast, at non-p53 regulated promoters, such as *ACTIN* and *POLR2,* H3K9me3 is not induced in ΔE1B-55k infected cells relative to mock (Supp. Fig 22 and 24). Basal H3K9me3 is decreased at these promoters inΔE1B-55k/ΔE4-ORF3 infected cells, suggesting that E4-ORF3 may also restrain global demethylase activity. We conclude that E4-ORF3 inactivates p53 by inducing *de novo* H3K9me3 heterochromatin silencing at

p53 target promoters. With access denied, p53 is powerless to activate the transcription of downstream effectors.

The induction of heterochromatin formation is still relatively poorly understood. Thus, a major question is how is E4-ORF3 directly involved in inducing repressive H3K9me3 heterochromatin at p53 target promoters? E4-ORF3 does not co-localize with p53 and forms a distinctive web-like structure in the nucleus (Supp. Fig 13). We show that E4-ORF3 demarcates the formation of *de novo* H3K9me3 heterochromatin domains inΔE1B-55k infected cells. E4-ORF3 is, for the most part, adjacent to H3K9me3, suggesting it acts as a novel platform that catalyses heterochromatin formation through transient or long-range interactions (Supp. Fig 25–27). Using high resolution confocal microscopy, we show that E4-ORF3 forms a continuous scaffold that specifies *de novo* heterochromatin assembly as it weaves through the nucleus (Fig 4b). These data demonstrate a direct role for E4-ORF3 in orchestrating H3K9me3 heterochromatin silencing at p53 target promoters. Furthermore, they reveal an extraordinary nuclear scaffold that either builds on existing architectural features that organize cellular DNA or is a novel viral construction that targets heterochromatin assembly at p53 target promoters.

Selective silencing of the p53 transcription program

These data beg the question as to the specificity of E4-ORF3 in silencing p53 targets. To determine the global consequences on cellular transcription, we performed genome-wide expression analyses on infected SAECs (Supp. Fig 28 and 29). These studies demonstrate that E4-ORF3 is an exclusive player in the global transcriptional changes induced upon viral infection. There are 1,730 overlapping genes that are similarly up or downregulated by a log fold change (FC) greater than two in bothΔE1B-55k and ΔE1B-55k/ΔE4-ORF3 *versus* mock, which reflect a common transcriptional program (Fig 5a). These global changes are associated with the cell cycle and E2F activation (Supplementary Table I and II). This is consistent with E1A mediated inactivation of RB 34 and recruitment of p300 and PCAF to induce active histone acetylation marks at the promoters of genes involved in cell growth, division and DNA synthesis 35,36. Thus, E4-ORF3 induced heterochromatin silencing, as well as the scaffold it forms throughout the nucleus, does not affect the global activation of cellular transcripts induced by viral infection.

To define the genes specifically targeted by E4-ORF3, we compared ΔE1B-55k/ΔE4-ORF3 *versus*ΔE1B-55k infected cells. E4-ORF3 prevents the transcriptional activation of 265 genes by a log fold change of two or more in ΔE1B-55k infected cells. To determine how many of these genes are likely to be regulated by p53, we used two criteria: the presence of consensus p53 DNA binding sites in their promoters and their induction upon treatment with the MDM2 antagonist, nutlin. A heat map of top transcripts differentially upregulated in response to ΔE1B-55k/ΔE4-ORF3 and nutlin includes well known p53 targets (MDM2, FAS, PIG3, TP53INP1, BTG2, LRDD) associated with growth inhibition and apoptosis, as well as novel targets (HRH1, RNASE7, JMJD1C) (Fig 5b and Supp. Fig 30). Of the 265 differentially upregulated genes, 73% are induced in response to nutlin and/or have predicted p53 binding sites (Fig 5c and Supplementary Table III–IV). A pathway analysis of E4-ORF3 regulated transcripts indicates that in addition to the p53 pathway, there is a

significant overrepresentation of genes associated with immune modulation as well as tissue/vascular remodeling (Supplementary Table V). These data suggest that E4-ORF3 may target p53 promoters as part of a general anti-viral transcriptional silencing program, which is consistent with the highly defective replication of ΔE1B-55k/ΔE4-ORF3 in primary cells (Supp. Fig 31).

Discussion and perspective

The conclusions of our study challenge the general assumption that p53 induction and phosphorylation is tantamount to p53 activity, which is the premise for several cancer therapies 11–13. Our data reveal a novel and dominant mechanism of p53 inactivation that acts *via* the targeted epigenetic silencing of p53 target promoters. We identify a viral protein, E4-ORF3, which appears to form a novel scaffold that weaves through the nucleus, directing SUV39H1/2 H3K9me3 heterochromatin assembly at p53 target promoters to silence p53 activated transcription in response to genotoxic and oncogenic stress (Fig 5d). Remarkably, this suppressive nuclear web selectively ensnares p53 and anti-viral genes while operating in the backdrop of global transcriptional changes that drive pathological cellular and viral replication.

There is a profound functional overlap between adenovirus early proteins and tumor mutations 37. Thus, a major question is if E4-ORF3 reflects or exhorts an existing cellular mechanism and nuclear structure that censors p53 transcriptional activity. Strikingly, all of the known targets of E4-ORF3, PML 38, the MRE11/RAD50/NBS1 (MRN) DNA damage/repair complex 39 and Tif1α 40 are subverted by tumor mutations. It is intriguing to speculate that E4-ORF3 physically integrates the inhibitory effects of several cancer pathway mutations, both known and yet to be discovered, which together have emergent functions 4 in silencing p53 activity. Similar to the discovery of p53 with a viral protein 2,3, E4-ORF3 provides a powerful dynamic probe with which to define critical cellular factors that induce *de novo* epigenetic silencing of p53 target promoters in somatic cells. This has important implications for understanding how high levels of wild-type p53 might also be inactivated in cancer as well as the dynamic mechanisms that induce aberrant epigenetic silencing of tumor suppressor gene loci. Finally, our identification of E4-ORF3 changes the fundamental definition of how p53 is inactivated in adenovirus infected cells, which is a critical mechanistic insight that could now enable the rational development of true p53 tumor selective adenoviral therapies.

Methods Summary

Cells were cultured and infected with established conditions 21,22. Protein lysates were analyzed by Western blotting 21,22. Real-Time Quantitative PCR (RT-QPCR) was used to quantify p53 targets 21, and normalized relative to 18S. For luciferase assays, U2OS cells were transfected and infected after 36 hours. 100 uM D-Luciferin was added 4 hours post infection (h.p.i.) and luminescence quantified every hour. Global gene expression was determined using Affymetrix Human Exon 1.0ST arrays and analyzed with Partek and Genomatix software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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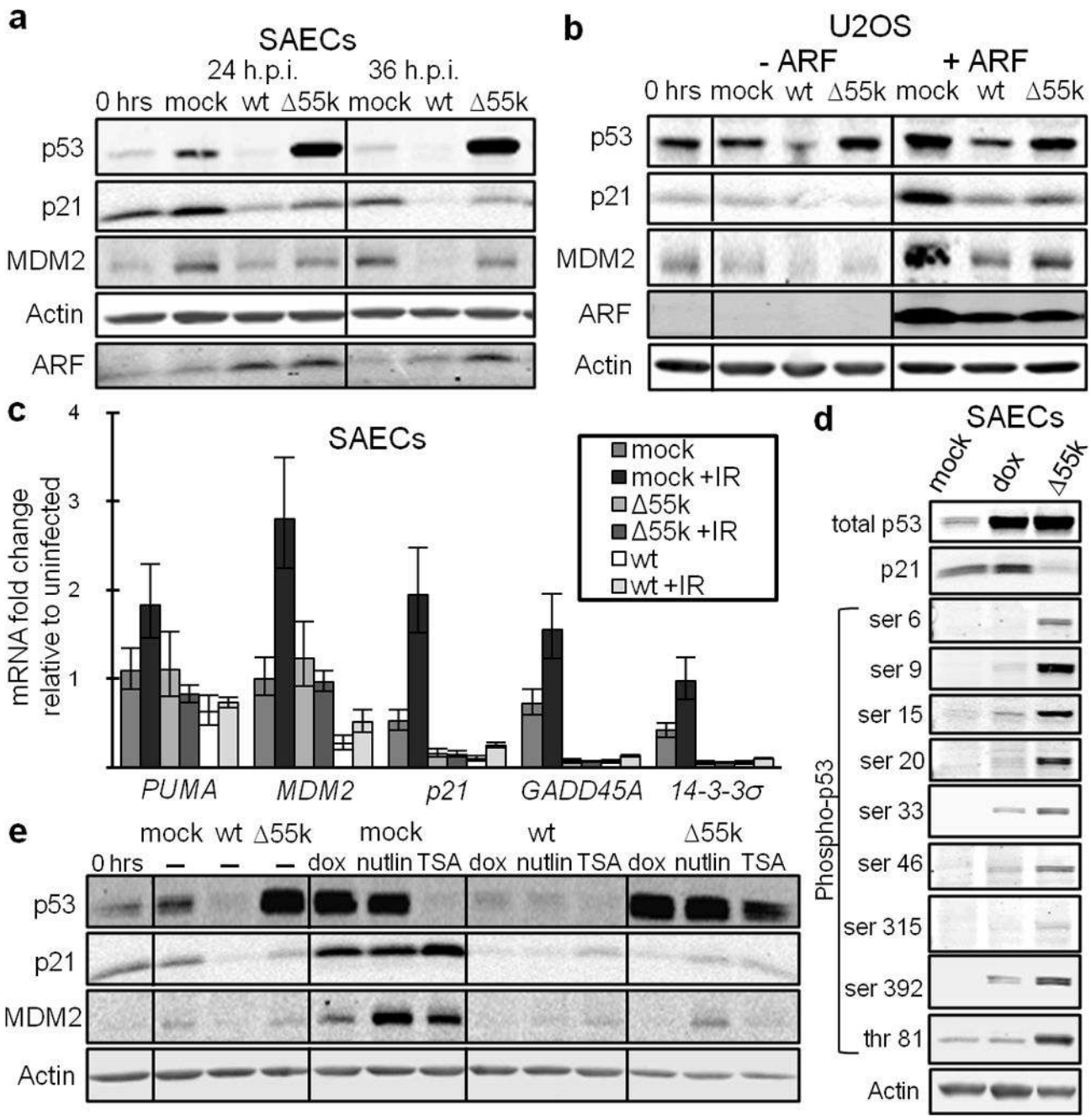

Fig 1. p53 is induced and phosphorylated in ΔE1B-55k infection but p53 activity is dominantly suppressed

a. SAECs were infected and protein lysates analyzed by immunoblotting. **b.** U2OS cells with inducible *ARF* were infected as indicated and analyzed for p53 levels and activation by immunoblotting. **c.** RT-QPCR of p53 transcriptional targets in infected SAECs (36 h.p.i.) plus/minus 10 Gy γ irradiation (IR). Error bars represent s.d. (n=3). **d.** Immunoblot of p53 protein phosphorylation in infected or doxorubicin (dox) treated SAECs (36 h.p.i.). **e.** Immunoblot of SAECs (36 h.p.i.) infected as indicated and treated with either control (−), dox, nutlin, or TSA at 24 h.p.i.

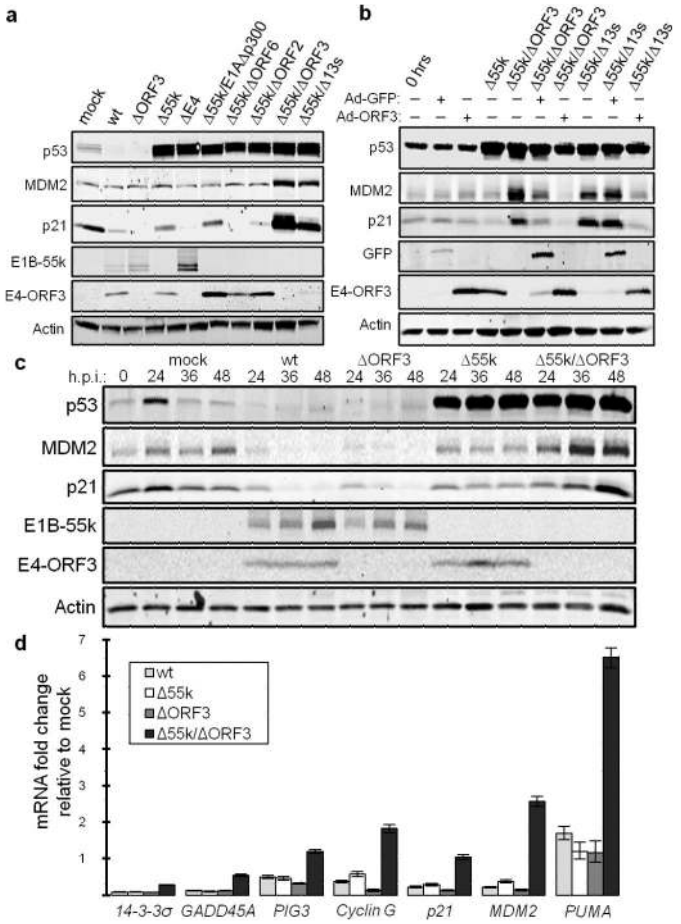

Fig 2. E4-ORF3 inactivates p53 independently of E1B-55k and p53 degradation

a. SAECs were infected with the indicated viruses (detailed description in Supp. Fig 8) and protein lysates (36 h.p.i.) analyzed for p53 activation by immunoblotting. **b.** SAECs were co-infected as indicated with either a GFP control virus (Ad-GFP, +) or a virus expressing E4-ORF3 (Ad-ORF3, +). Protein lysates (36 h.p.i.) were analyzed for p53 activation by immunoblotting. **c.** SAECs were infected and harvested over a 48hr time course as indicated and analyzed for p53 activation by immunoblotting. **d.** RT-QPCR of p53 transcriptional targets in infected SAECs at 36 h.p.i. Error bars represent s.d. (n=3).

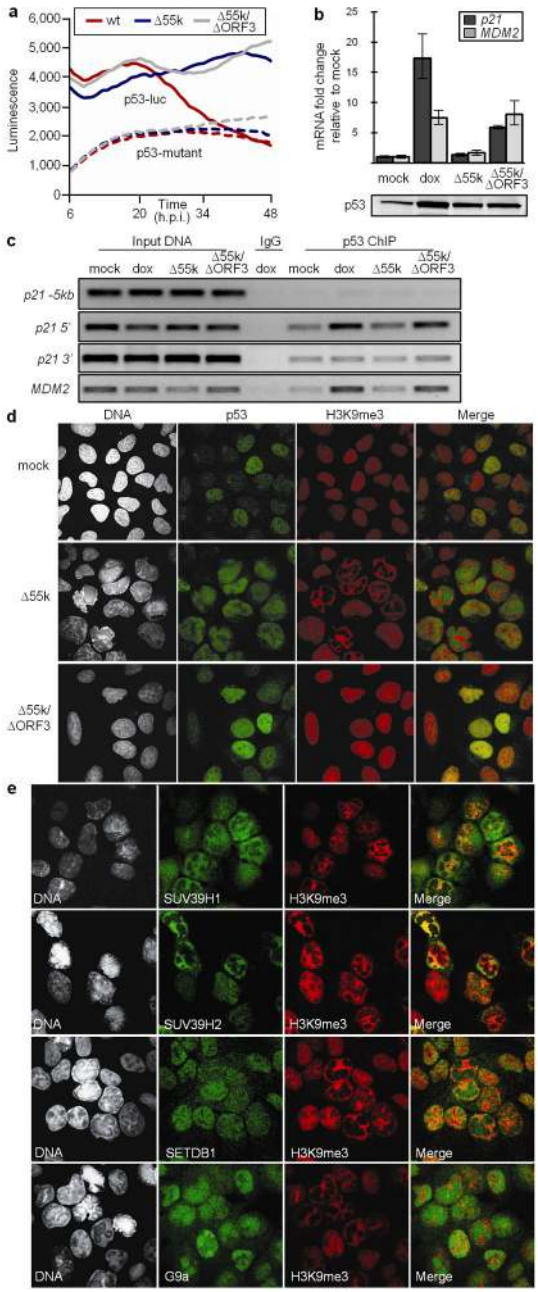

Fig 3. E4-ORF3 induces heterochromatin formation and prevents p53-DNA binding at endogenous promoters

a. U2OS cells were transfected with p53-luc (solid line) or p53-mutant (dashed line) luciferase plasmids and infected with indicated viruses. Luminescence is plotted against time. **b.** and **c.** U2OS cells were infected as indicated or treated with doxorubicin. **b.** p53 induction was analyzed by immunoblotting and p53 transcriptional targets quantified by RT-QPCR (36 h.p.i.). Error bars represent s.d. (n=3) **c.** p53 ChIPs were analyzed by semi-quantitative PCR for *p21* and *MDM2* promoter sequences. **d.** p53 (green) and H3K9me3 (red) immunofluorescence of infected U2OS cells (36 h.p.i.). **e.** Co-localization of

SUV39H1, SUV39H2, SETDB1 and G9a (green) with H3K9me3 (red) in Δ55k infected U2OS cells (36 h.p.i.).

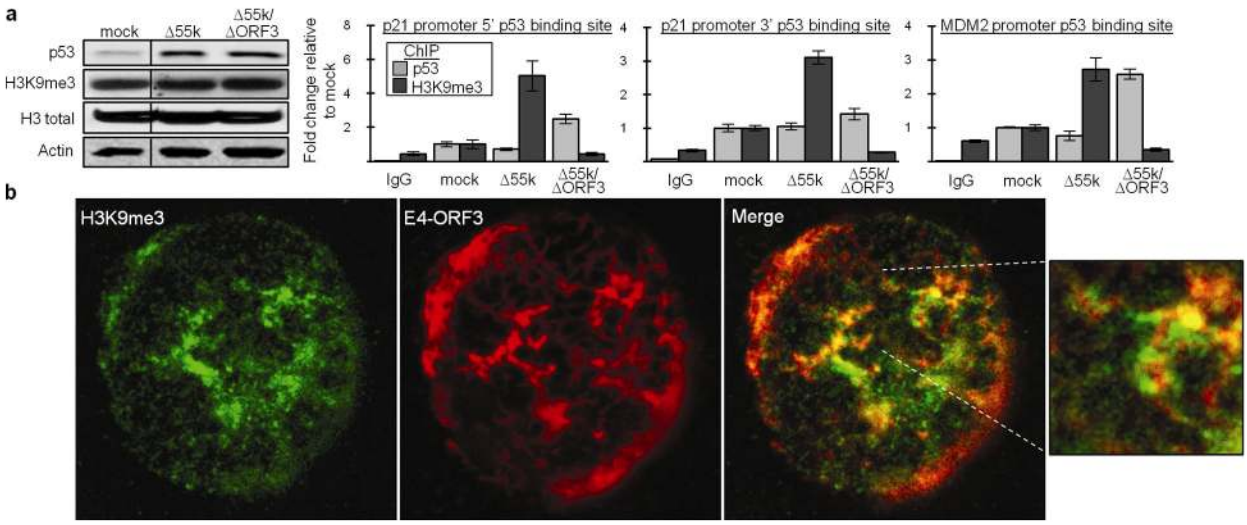

Fig 4. E4-ORF3 forms a nuclear scaffold that specifies heterochromatin assembly and H3K9 trimethylation at p53 target promoters

a. Protein lysates from infected U2OS cells (36 h.p.i.) were analyzed for total Histone H3 or H3K9me3 levels by immunoblotting. H3K9me3 and p53 ChIPs were quantified by RT-QPCR, normalized relative to input DNA and plotted as fold change relative to mock. Error bars represent s.d. (n=2) **b.** H3K9me3 (green) and E4-ORF3 (red) localization in Δ55k infected SAECs (36 h.p.i.) was visualized by immunofluorescence. A high resolution confocal slice (0.3μm) through the nucleus is shown with a magnified section of E4-ORF3 and associated heterochromatin domains on the far right.

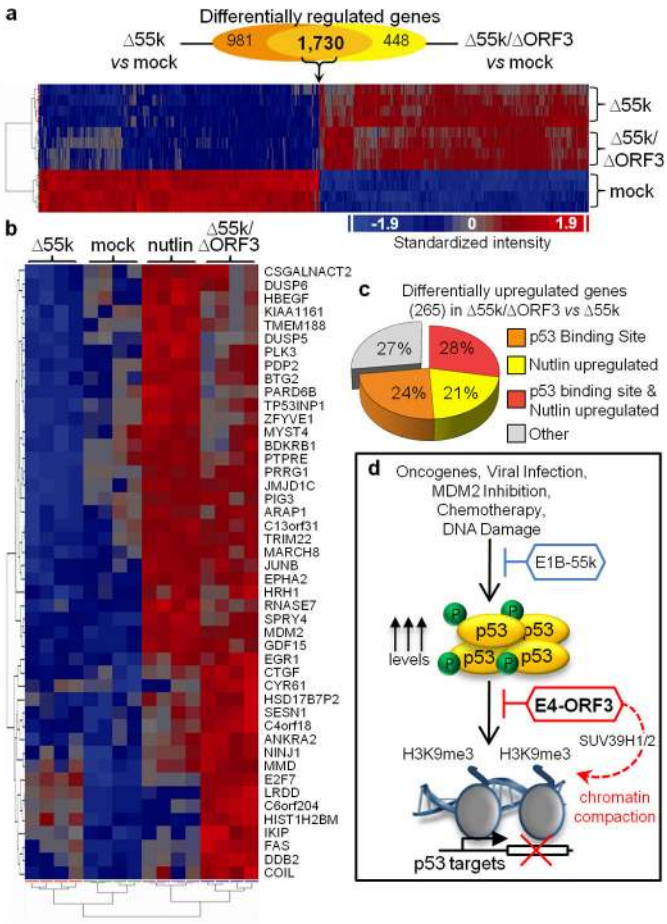

Fig 5. p53 transcriptional targets are silenced selectively in the backdrop of global transcriptional changes that drive oncogenic cellular and viral replication

Affymetrix global gene expression analyses of SAECs**a.** Heat map of the 1,730 overlapping differentially regulated genes (log FC>2 or <−2 with a false discovery rate (FDR) of 0.05) between Δ55k/ΔORF3 andΔ55k *versus* mock infected SAECs (36 h.p.i.). **b.** Unsupervised hierarchical clustering of 46 top differentially upregulated transcripts in both Δ55k/ΔORF3 infection and nutlin treatment. **c.** Pie-chart depicting the percentage of upregulated transcripts (log FC>2 and FDR of 0.05) in Δ55k/ΔORF3 *versus*Δ55k that have predicted p53 transcription factor binding sites and/or induced by a log FC>1.5 in response to nutlin. **d.** Summary and model.