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KMTase Set7/9 is a critical regulator of E2F1 activity upon genotoxic stress

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During the recent years lysine methyltransferase Set7/9 ((Su(var)-3–9, Enhancer-of-Zeste, Trithorax) domain containing protein 7/9) has emerged as an important regulator of different transcription factors. In this study, we report a novel function for Set7/9 as a critical co-activator of E2 promoter-binding factor 1 (E2F1)-dependent transcription in response to DNA damage. By means of various biochemical, cell biology, and bioinformatics approaches, we uncovered that cell-cycle progression through the G1/S checkpoint of tumour cells upon DNA damage is defined by the threshold of expression of both E2F1 and Set7/9. The latter affects the activity of E2F1 by indirectly modulating histone modifications in the promoters of E2F1-dependent genes. Moreover, Set7/9 differentially affects E2F1 transcription targets: it promotes cell proliferation *via* expression of the *CCNE1* gene and represses apoptosis by inhibiting the *TP73* gene. Our biochemical screening of the panel of lung tumour cell lines suggests that these two factors are critically important for transcriptional upregulation of the *CCNE1* gene product and hence successful progression through cell cycle. These findings identify Set7/9 as a potential biomarker in tumour cells with overexpressed E2F1 activity.
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Lysine methylation of non-histone proteins has recently emerged as a novel regulatory mechanism to control protein functions.[1–4] Set7/9 ((Su(var)-3–9, Enhancer-of-Zeste, Trithorax) domain containing protein 7/9) is a founding member of the family of non-chromatin lysine methyltransferases (KMTases). Set7/9 was initially identified as a monomethylase of histone H3 lysine 4 (H3K4) *in vitro*.[4,5] However, we and others showed that the recombinant Set7/9 failed to target nucleosomes for methylation,[6–8] suggesting that Set7/9 functions as a factor-specific KMTase. There have been several non-histone proteins reported as the substrates for Set7/9, including TAF10 (TATA box binding protein (TBP)-associated factor, 30 kDa),[9] oestrogen receptor α (ERα),[10] RelA,[11] PCAF (P300/CBP-associated factor),[12] Stat3,[13] Yap,[14] and Suv39h1.[15] However, in most cases the functional significance of this methylation is still not clear. The best-studied targets of Set7/9-mediated methylation are p53[16] and E2 promoter-binding factor 1 (E2F1),[17] transcription factors involved in regulation of DNA damage response (DDR).

In response to genotoxic stress cancer cells undergo cell-cycle arrest either in G1/S or G2/M or in both checkpoints. The presence of intact p53 in cancer cells mediates transient G1/S checkpoint arrest,[18,19] which allows cells to repair the damaged DNA before replication or, if the amount of damage is insurmountable, drives cells into apoptosis.[20,21]

On the contrary, the activity of the E2F family transcription factors, especially E2F1, drives cells from the G1/S block to mitosis.[22,23] Transcriptional activity of E2F1, in turn, is repressed by the retinoblastoma protein (Rb) family proteins.[24–26] The latter are inactivated by phosphorylation mediated by cyclin-dependent kinase (Cdk2) and Cdk4/Cdk6 cell-cycle kinases.[27–29] The E2F1 transcription factor drives cell-cycle progression by activating the transcription of *CCNE* gene, whose product is essential for Cdk2 kinase activity.[30] Cdk2/cyclin E and Cdk4/6 partnered with various forms of cyclin D are the principal kinases required for replication of DNA.[31,32] In addition, E2F1 regulates a number of genes involved in DNA repair, chromosomal stability, and apoptosis.[33,34]

Importantly, E2F1 induces transcription of p53,[35] whereas p53 represses the activity of E2F1, thus forming a negative feedback loop.[23,36]

The functional outcome of Set7/9-mediated methylation of p53 and E2F1 is diametrically opposite for these two proteins: methylation of p53 on K372 stabilises the protein by inducing its subsequent acetylation,[37] yet methylation of E2F1 on

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Abbreviations: DDR, DNA damage response; Set7/9, (Su(var)-3–9, Enhancer-of-Zeste, Trithorax) domain containing protein 7/9; E2F1, E2 promoter-binding factor 1; KMTase, lysine methyltransferase; TAF10, TATA box binding protein (TBP)-associated factor, 30 kDa; ERα, oestrogen receptor; PCAF, P300/CBP-associated factor; Rb, retinoblastoma protein; Cdk, cyclin-dependent kinase; BrdU, bromodeoxyuridine; siRNA, small interfering RNA; shRNA, short hairpin RNA; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase; HSP90, heat shock protein 90; PARP, poly (ADP-ribose) polymerase; RPMI, Roswell Park Memorial Institute; FBS, foetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; RIPA, radioimmunoprecipitation assay; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorting

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K185[17] on the contrary, interferes with acetylation, promotes its ubiquitylation and subsequent degradation *via* the 26S proteasome.[38,39] It has also been shown that, in addition to p53 and E2F1, Set7/9 affects other substrates *via* regulating their protein stability either positively (ERα)[10] or negatively (DNMT1, RelA).[40,41]

In this study, we report a novel function for Set7/9 as a critical co-activator of E2F1-dependent transcription in response to DNA damage. By means of various biochemical, cell biology, and bioinformatics approaches, we conclude that cell-cycle progression through G1/S phase of tumour cells upon DNA damage is defined by the threshold of expression of both E2F1 and Set7/9. Our screening of the panel of lung tumour cell lines suggests that these two factors are critically important for transcriptional upregulation of the *CCNE* gene product and hence successful exit from the G1/S checkpoint arrest. These findings identify Set7/9 as an attractive target for pharmacological inhibition with small molecules.

Results

Inactivation or ablation of Set7/9 results in G1/S arrest upon DNA damage. Since Set7/9 affects both p53 and E2F1 transcription factors whose activities regulate cell-cycle regulation and apoptosis, we reasoned that Set7/9 also must have a role in execution of cell-cycle checkpoints in response to DNA damage. To test this hypothesis, we analysed cell-cycle distribution of non-small human lung carcinoma cells, H1299, with short hairpin RNA (shRNA)-mediated knockdown of Set7/9 (H1299 Set9KD), along with parental cells transfected with control shRNA (H1299) (Figure 1a). These cells are negative for p53, but express E2F1. To induce DNA damage, cells were treated with doxorubicin for the indicated periods of time. Expectedly, H1299 cells exhibited cell-cycle arrest mostly in S and G2 phases, which is typical for p53$^-$ cells that lack G1/S checkpoint.[18] On the contrary, H1299 Set9KD cells displayed a stronger G1/S-phase arrest compared with the control cells.

We also wanted to expand our observations on p53$^+$ cells. We chose p53$^+$ human osteosarcoma U2-OS cells, which exhibit a more pronounced arrest in G2/M in response to DNA damage due to overexpression of Wip1 phosphatase.[42] Therefore, U2-OS represents a convenient model to study effects of Set7/9 on G1/S arrest after DNA damage in the presence of p53. Thus, we generated cells expressing (U2OS control) or lacking Set7/9 (U2OS Set9KD). To examine the role of lysine methylation activity of Set7/9 in cell cycle, we generated shRNA-resistant catalytically active and inactive (Set7/9wt and Set7/9mut, respectively) expression constructs and overexpressed them in U2OS Set9KD cells (Figure 1b). Whereas U2-OS control cells showed mostly G2/M arrest, cells lacking Set7/9 were preferentially arrested in G1/S. Moreover, only wild-type Set7/9, but not Set7/9 mut, was able to restore the original phenotype of parental cells. Importantly, ectopic Set7/9 wild-type and the mutant proteins were expressed at the comparable levels (Figure 1c).

Notably, an accumulation of Set7/9$^-$ cells in G1/S after genotoxic stress was observed in various cell lines irrespectively of the p53 status and source of DNA damage (doxorubicin, X-rays, or UV) (Supplementary Figures 1–3).

Our results suggested two plausible scenarios: (i) the attenuation of Set7/9 expression or its activity compromised the G2/M checkpoint arrest and hence, cells were accumulated at the G1/S boundary after the round of mitosis; (ii) Set7/9-deficient cells were never able to exit G1 after DNA damage and thus could not proceed through S phase to G2. To discriminate between these possibilities, we performed several experiments. First, using phospho-serine 10 in histone H3 (H3-S10-phos) staining as a marker of mitosis, we counted at different time points the number of mitotic cells in U2-OS control and U2-OS Set7/9KD cells after their treatment with the indicated doses of doxorubicin (Figure 1d). A much lower number of U2-OS Set7/9KD cells entered mitosis at 0.5 μM of doxorubicin treatment compared with the control cells. These data suggest that Set7/9$^-$ cells are likely arrested in G1/S. To confirm this, we stained U2-OS and U2-OS Set7/9KD cells with bromodeoxyuridine (BrdU) after treatment with doxorubicin (Figure 1e). BrdU is incorporated only in the newly synthesised DNA and thus manifests the S-phase transition. While U2-OS control cells resume their cell cycle after 24 h post doxorubicin treatment, U2-OS Set7/9KD cells were halted in G1 and G2 phases without any visible population of cells that newly entered S phase. This suggests that the ablation of Set7/9 not only imposes G1/S arrest but also blocks the transition through S phase. Collectively, these results strongly argue that lysine methylation by Set7/9 has an important role in regulating cell-cycle progression upon DNA damage.

Set7/9 regulates the level of cyclin E expression. It is well established that the driving force behind the transition of cells from G1 to S phase is the kinase activity of the Cdk2/cyclinE and Cdk4/cyclin D complexes. Accordingly, we tested the ability of Cdk2 and Cdk4 immunoprecipitated from U2-OS control and Set7/9KD cells to phosphorylate histone H1, a common substrate for various kinases.[43] Since p53 is known to inhibit the activity of various cdks *via* p21/CIP,[44] we also examined U2-OS cells with knockdown of p53 expression (U2-OS p53KD) (Figure 2a). We found that control U2-OS cells displayed high cdk2 activity, which decreased upon DNA damage. However, in Set7/9KD cells the activity of Cdk2 was somewhat lower even in the absence of DNA damage and was almost ameliorated after 24 h of doxorubicin treatment. p53-deficient cells showed intermediate levels of Cdk2 activity, higher than in Set7/9KD cells, but lower than in the control cells (Figure 2a, upper). This is due to their faster transition through G1/S phase, where the activity of cdk2 is the highest.[45] On the contrary, the activity of cdk4, another principal kinase that drives G1/S transition, after DNA damage was comparable in both U2-OS control and Set7/9KD cells (Figure 2a, lower). Put together, these results suggest that Set7/9 specifically modulates the activity of the Cdk2/cyclin E complex upon DNA damage.

Kinase activity of the Cdk2 complex depends on the protein levels of both Cdk2 and cyclin E. Thus, we tested the levels of Cdk2 and cyclin E expression in control, Set7/9KD, and p53KD U2-OS cells before and after DNA damage (Figure 2b). We found that the levels of cdk2 expression were comparable in all three cell lines, whereas the levels of cyclin E differed drastically between the cell lines (Figure 2b, middle

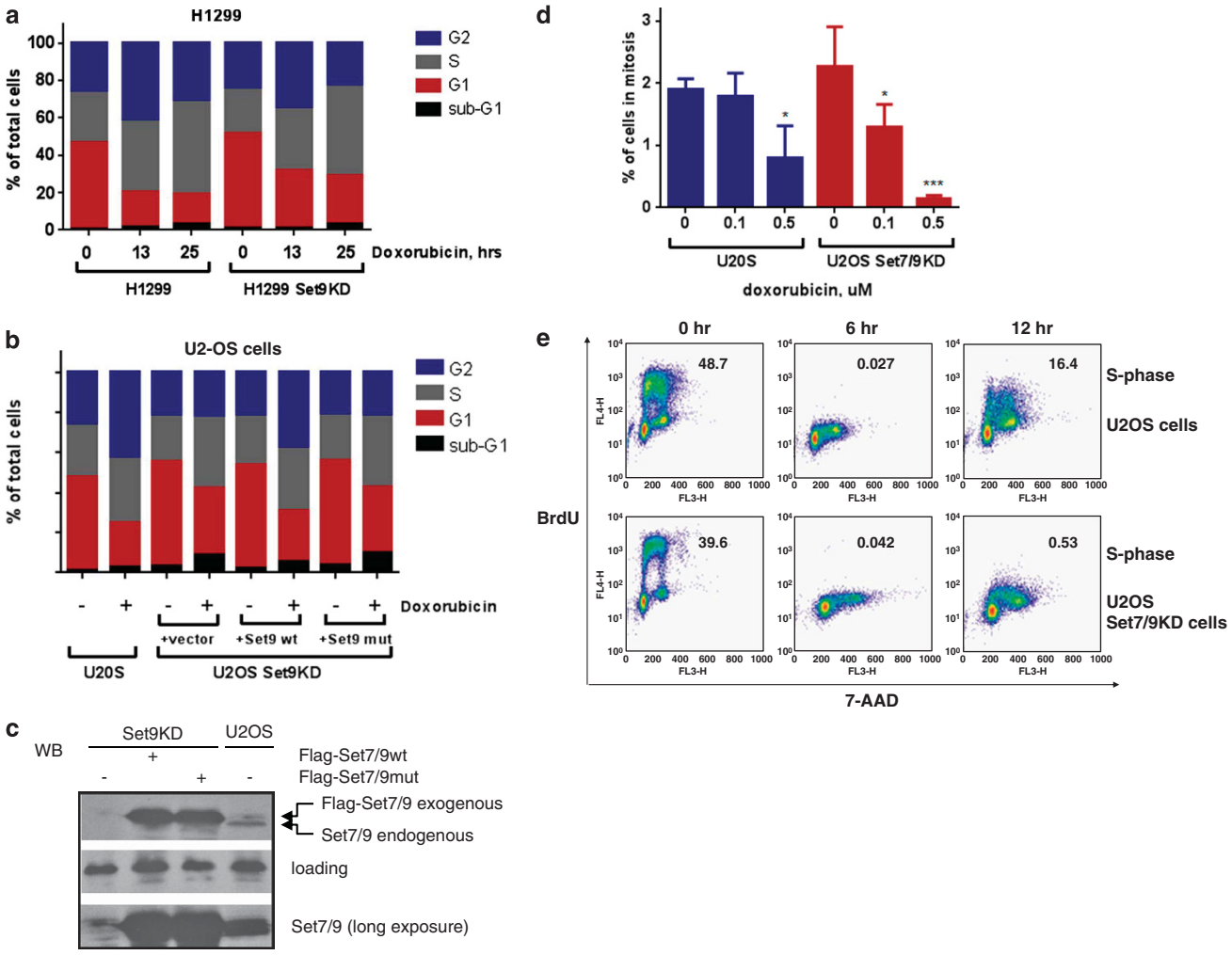

Figure 1 Set7/9 affects cell-cycle distribution of tumour cells upon DNA damage. (**a**) Human H1299 lung adenocarcinoma cells stably expressing shRNA against Set7/9 or control shRNA were treated with doxorubicin for the indicated periods of time. The resulting cells were fixed, stained with propidium iodide and analysed for cell-cycle distribution. (**b**) Human osteosarcoma U2-OS cells were treated or non-treated with 0.5 μM doxorubicin for 14 h and analysed for cell-cycle distribution as described above. In parallel, U2-OS stably expressing shRNA against Set7/9 (U2OS Set9KD) were transfected with an empty vector, or the one expressing Set7/9wt, or Set7/9 mut (H293A). Transfected cells were treated as control U2OS cells and subjected to cell-cycle analysis. Statistical analysis was done by one-way ANOVA. All treated samples were compared with the untreated ones. Also, U2-OS Set7/9KD cells were compared with U2-OS control cells at 0 time point. Abbreviations: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; $^{****}P<0.0001$. (**c**) Western blot analysis of ectopic expression of Set7/9 wild-type and H279A mutant in U2-OS Set7/9KD cells. Levels of endogenous Set7/9 in non-transfected U2-OS and U2-OS Set7/9KD cells are also shown. Actin served as a loading control. (**d**) U2-OS cells expressing control or Set7/9-specific shRNA were pulse-treated with the indicated amounts of doxorubicin for 2 h followed by incubation for 16, 24, and 48 h. At each time point the number of mitotic cells was determined for both cell lines using phospho-S10-H3 staining. The total number of cells was also calculated. The percentage of mitotic cells was calculated as an average of mitotic cells obtained for each time point divided by the total number of cells in each case. (**e**) U2-OS control (U2OS cells) and U2-OS cells expressing shRNA-Set7/9 (U2OS Set9KD cells) were treated with 0.5 μM doxorubicin for the indicated times followed by labelling with BrdU. Subsequently, cells were permeabilised, treated with DNAse, stained with anti-BrdU antibodies conjugated to FITS, followed by staining with 7-AAD to visualise DNA. The resulting samples were analysed by FACS. The numbers shown indicate the percentage of cells in S phase

panel). While control U2-OS cells accumulated cyclin E after DNA damage, very little accumulation was observed in DNA damage-treated Set7/9KD cells. Expectedly, p53 stabilisation in response to DNA damage was compromised in Set7/9KD cells, as published previously.[6] Notably, Cdk4/cyclin D1 levels in the control and Set7/9KD cells were comparable, indicating that this kinase complex did not affect the arrest of Set7/9KD cells in G1/S phase (Figure 2c). Similarly, a low level of cyclin E was detected in p53KD cells both before and after DNA damage.[46] The latter effect can be explained by the fact that the cellular amount of cyclin E decreases after the passage of cells through S phase. Note, that $p53^{-}$ cells undergo G2/M arrest in response to double-strand breaks and thus pass through G1/S phase quickly.

In agreement with previously published results Set7/9KD cells contained increased levels of E2F1 both before and after DNA damage compared with the levels of E2F1 in U2-OS control cells, where its expression is repressed by p53[47] (Figure 2b). Accordingly, E2F1 levels were higher in p53KD cells compared with the control cells (Figure 2b).

Given that the constitutive knockdown of Set7/9 may affect gene expression indirectly, we generated an inducible Tet-on

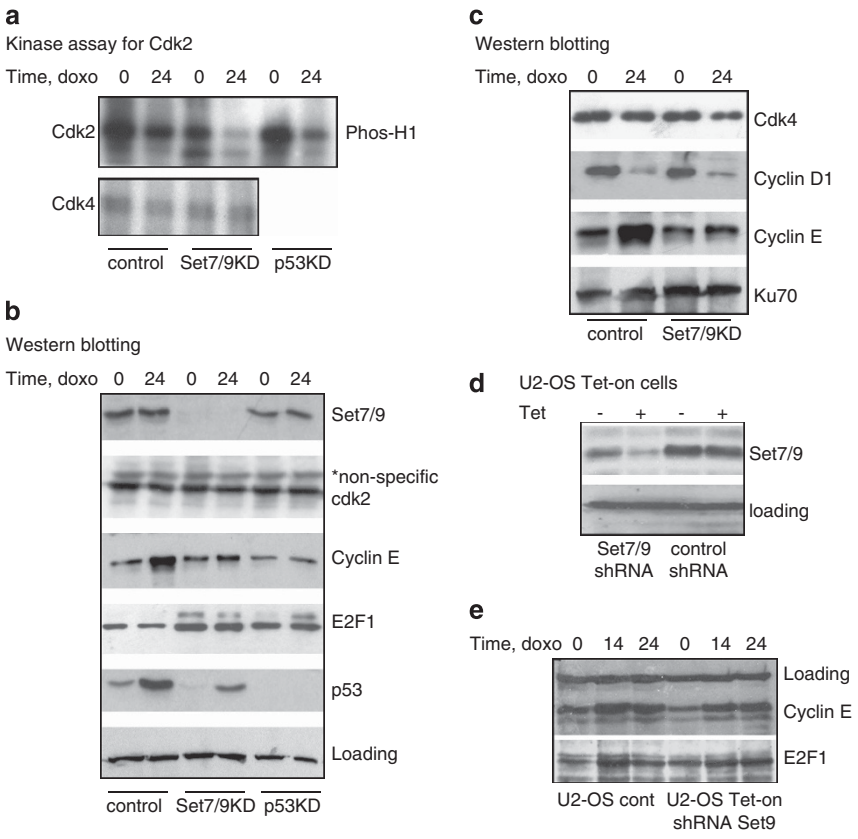

Figure 2 Set7/9 controls Cdk2 activity by affecting the expression of cyclin E. (**a**) U2-OS parental control cells and cells with knockdown expression of Set7/9 and p53 were treated with doxorubicin for the indicated times and subject to immunoprecipitation with anti-cdk2 or anti-cdk4 antibodies. The resulting immunopurified Cdk2/cyclin E and cdk4/cyclin D complexes were analysed for their ability to phosphorylate H1. (**b**) Western blot analysis of the cell lines indicated above for the levels of expression of Set7/9, cdk2, cyclin E, E2F1, and p53. Actin signal was used as a loading control. (**c**) Western blot analysis of U2-OS and U2-OS Set7/9$^-$ cells (Set7/9KD) treated with doxorubicin for the indicated times and subject for immunoblotting with cdk4, cyclin D1, and cyclin E antibodies. Ku70 was used as a normalisation control. (**d**) Tet-on U2-OS cells with inducible expression of control shRNA or shRNA against Set7/9 were induced with 2 μg/ml doxycycline or tetracyclin (Tet) for 3 days before assessing the efficiency of knockdown of Set7/9 by western blotting. Actin signal was used as a loading control. (**e**) The abovementioned cells were treated with doxorubicin and analysed for the levels of cyclin E and E2F1 by western blotting

U2-OS cell line and found that, similar to our previous results, activation of Set7/9-specific shRNA attenuated the level of cyclin E expression in response to doxorubicin compared with control shRNA-expressing cells (Figures 2d and e). The kinetics of E2F1 accumulation after DNA damage in U2-OS control cells differed from that in U2-OS Set7/9$^-$ cells. In the control cells, E2F1 levels declined by 24 h due to repression mediated by p53. In contrast, the level of E2F1 in Set7/9$^-$ cells, where the function of p53 is compromised, peaked at 24 h (Figure 2e).

Set7/9 regulates E2F1-mediated transcription. Next, we wanted to find out whether the attenuation of cycin E expression in Set7/9KD cells occurs on the level of protein stability or on the level of transcription. To address this question, we employed luciferase reporter assay using the cyclin E promoter luciferase construct. Since E2F1 is the major regulator of *CCNE* transcription we also used a cyclin E promoter deletion mutant construct that lacks E2F1 binding sites (Figure 3a). As shown in Figure 3, deletion of the E2F1 responsive element from the cyclin E promoter (cycE mut) luciferase construct resulted in a significant attenuation of transactivation in both H1299 and U2-OS cells compared with wild-type promoter. DNA damage increased transcription of cyclin E promoter in both H1299 and U2-OS cells (Figures 3c and e). This is consistent with the fact that E2F1 is activated by DNA damage.[23] In contrast, abrogation of Set7/9 expression in both cell types resulted in a significant reduction of cyclin E transcription.

To validate our results of luciferase assays, we assessed the effect of Set7/9 on expression of the endogenous *CCNE1* gene in U2-OS and U2-OS Set7/9KD cells (Figure 3f). In addition, we also examined expression of another target of E2F1, *TP73* (Figure 3g). Transcription of CCNE1 was elevated upon DNA damage in control cells, but did not change in Set7/9 KD cells, supporting the notion that Set7/9 is important for expression of cyclin E. In contrast with *CCNE1*, transcription of *TP73* was enhanced by the ablation of Set7/9, suggesting that Set7/9 may regulate E2F1 target genes differently. Collectively, these results indicate that Set7/9 is required for transcriptional activation of *CCNE1* by E2F1 in response to DNA damage.

Next, we thought to examine whether E2F1 together with Set7/9 contributes to the regulation of cyclin E and p73

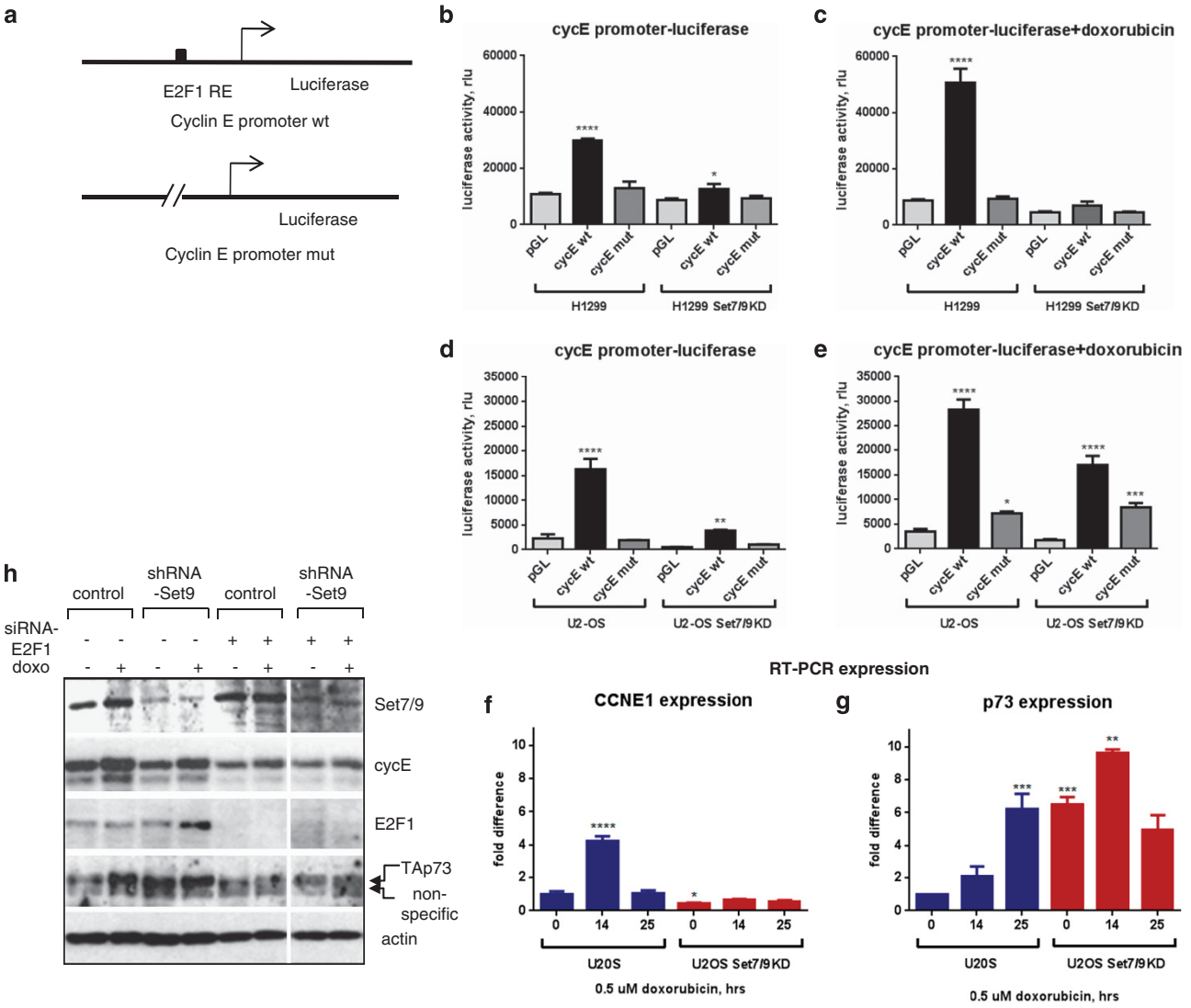

Figure 3 Set7/9 is a transcriptional co-regulator of E2F1. (**a**) The scheme of luciferase reporter plasmids bearing the cyclin E promoter fragments either containing E2F1 response element (E2F1 RE) (cycE wt) (upper), or lacking it (cycE mut) (lower) is shown. (**b** and **c**) H1299 or H1299 Set7/9^{-} (Set9KD) cells transfected with either wild-type or mutant cycE reporter plasmid were not treated or treated with 0.5 μM doxorubicin, respectively. Following incubation, cells were lysed and assayed for luciferase activity. The normalisation of efficiency of transfection was carried out based on the levels of activity of transfected beta-galactosidase. (**d** and **e**) U2-OS control (U2-OS) or U2-OS Set7/9^{-} (Set9KD) cells transfected as above were not treated or treated with doxorubicin as described above to assay for luciferase activity. (**f** and **g**) U2-OS control (U2-OS) or U2-OS Set7/9^{-} (Set9KD) cells treated with doxorubicin for the indicated periods of time were analysed by RT-PCR for the levels of expression of *CCNE1* (**f**) and *TP73* (**g**) genes. Expression levels of actin were used for normalisation. Statistical analysis was done by one-way ANOVA. Cells that expressed cyclin E promoter constructs were compared with the ones that expressed pGL2 empty vectors. Also, U2-OS Set7/9KD and U2-OS control cells were compared. Abbreviations are the same as in Figure 1d. (**h**) E2F1 and Set7/9 regulate cyclin E and p73 expression on the protein level. U2-OS control and Set7/9KD cells were transiently knocked down for E2F1 by specific siRNA. The efficiency of Set7/9 and E2F1 knockdowns was controlled by westerns. The levels of cyclin E and p73 expression before and after doxorubicin treatment (doxo) were measured by western blotting using specific antibodies. Note, that the antibody against p73 consistently produced non-specific background (marked as non-specific)

expression on the protein level. To address this question, we transiently knocked down E2F1 by small interfering RNA (siRNA) both in U2-OS control and in Set7/9KD cells and measured the levels of cyclin E and p73 expression before and after DNA damage (Figure 3h). We found that in the absence of E2F1 the levels of cyclin E were lower in both U2-OS control and Set7/9KD cells before and after DNA damage, suggesting that E2F1 is the principal regulator of cyclin E expression. Ectopic expression of Set7/9 in E2F1-knockdown cells (data not shown) did not restore the level of cyclin E, indicating that Set7/9 operates upstream of E2F1.

Set7/9 regulates E2F1 binding and histone modifications on the target promoters. To investigate how Set7/9 affects the transcriptional activity of E2F1, we performed a series of chromatin immunoprecipitation (ChIP) assays on promoters of the *CCNE1* and *TP73* genes (Figure 4). Expectedly, DNA damage increased E2F1 binding to promoters of both genes. However, Set7/9 affected the DNA binding ability of E2F1 only moderately (Figure 4a, right and left panels), suggesting that the regulation occurs not on the level of DNA binding but on the level of chromatin modifications. Set7/9 did not affect the levels of K4 mono-methylation of histone H3 (H3K4Me1)

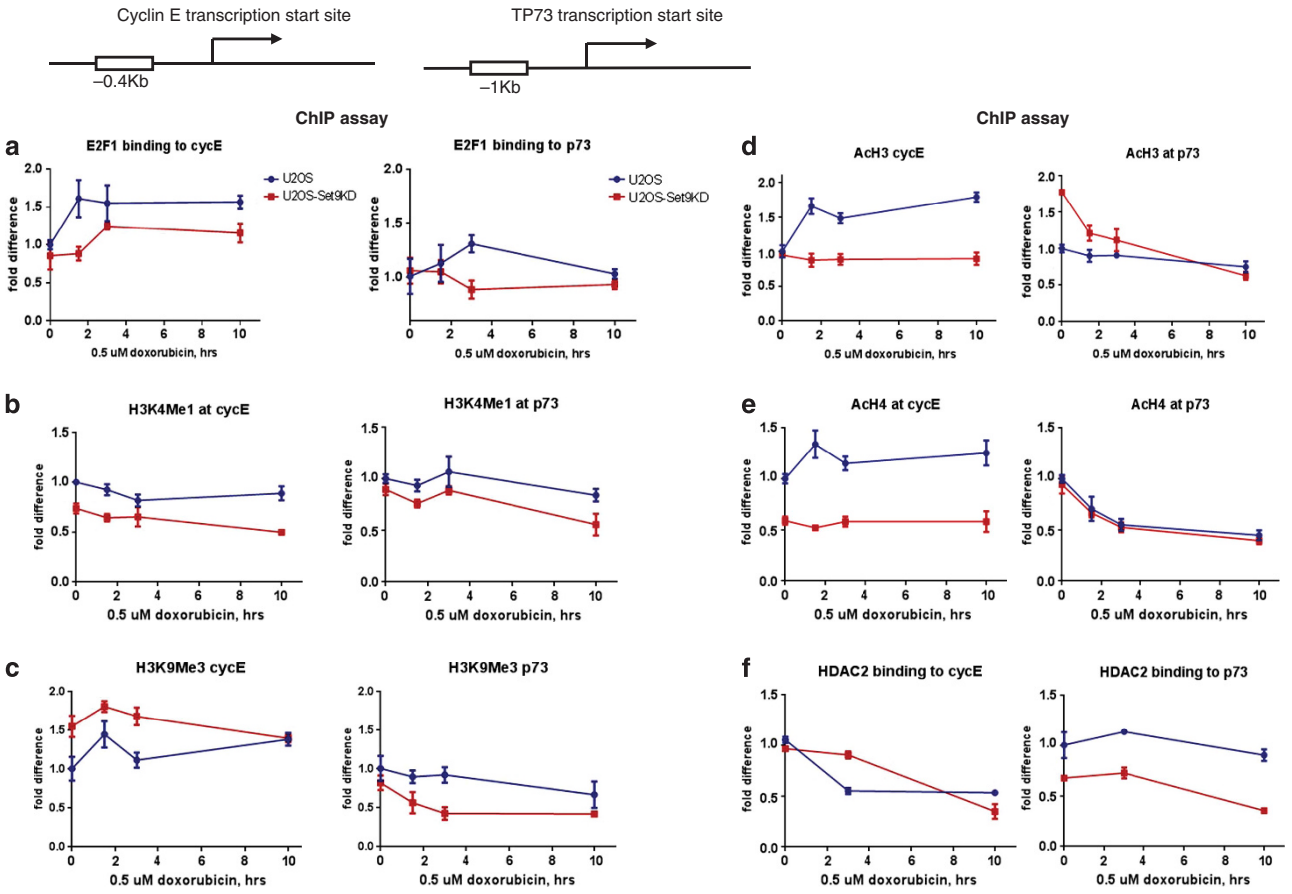

Figure 4 Set7/9 affects histone modifications in the promoters of E2F1-dependent genes. The schemes of amplicons specific for the promoter regions of CCNE1 and TP73 relative to the start site are shown. ChIP assay for E2F1 (**a**), H3K4 mono-methylation (**b**), H3K9 tri-methylation (**c**), AcH3 (**d**), AcH4 (**e**), and HDAC2 (**f**) at the promoters of *CCNE1* (left) and *TP73* (right) genes. PCR signal in the untreated U2-OS control cells was arbitrary set as 1. Time periods of doxorubicin treatment are indicated. Blue lines denote U2-OS wild-type cells and the red ones –Set7/9 KD cells

(Figure 4b), supporting the notion that Set7/9 is not able to methylate chromatin. Interestingly, K9 tri-methylation of histone H3 (H3K9Me3), which is a repressive chromatin mark, was elevated for the promoter of CCNE1 in Set7/9KD cells compared with control U2-OS cells (Figure 4c, left), thus correlating with its decreased transcription in Set7/9$^-$ cells (Figure 3f). The *TP73* gene, which, in contrast to CCNE1, is repressed in control cells but becomes de-repressed in Set7/9KD cells (Figure 3g), displayed reverse correlation, that is, the level of H3K9Me3 was higher in the control cells compared with Set7/9$^-$ cells (Figure 4c, right). Next, we tested the dynamics of chromatin activating modifications, such as acetylation of histone H3 (AcH3) and H4 (AcH4). The levels of AcH3 and AcH4 signals on the CCNE1 promoter were increased by DNA damage and were higher in the control compared with Set7/9KD cells (Figures 4d and e, left panels). On the contrary, DNA damage attenuated both AcH3 and AcH4 levels on the TP73 promoter (Figures 4d and e, right panels). Moreover, the initial level of AcH4 in Set7/9KD cells was higher in Set7/9 KD compared with U2-OS control cells (Figure 4d, right). These data paralleled the binding kinetics of histone deacetylase 2 (HDAC2) to CCNE1 and TP73 promoters (Figure 4f, compare left and right panels). HDAC2 binds HDAC1 to form a repressive complex to deacetylate histones.[48] Taken together, the results of ChIP assay suggest that Set7/9 differently regulates transcription of E2F1-dependent genes by modulating the levels of chromatin modifications.

***In silico* bioinformatics analysis uncovers the E2F1-Set7/9 axis in lung tumour.** It is known that E2F1 can have both positive and negative roles in tumour progression, by either forcing the proliferation, or inducing apoptosis, respectively.[49] Therefore, the overall output of the E2F1 function may differ depending on a specific type of cancer.[50] Data mining of the literature coupled with the bioinformatics approach[51,52] have established E2F1 as an oncogene in lung cancers (Eymin *et al*.[53] and Huang *et al*.[54] Supplementary Data; Supplementary Figure S4 and data not shown). Therefore, we performed our next experiments using lung tumour cell lines.

Since Set7/9 negatively regulated TP73 expression, we questioned whether this negative correlation affected the survival of patients with lung cancer. Thus, we implemented a statistical procedure, which divided the patients into two cohorts. The first one was enriched with positive correlation between expression levels of Set7/9 and TP73, while all the other patients formed the second cohort. To identify statistical differences in survival outcome between these two groups,

the R statistical package (R Foundation for Statistical Computing, Vienna, Austria) was used to perform statistical tests and to derive the *P*-value (for full details of the procedure, see Celardo *et al*.[55] Two different lung cancer data sets of patients with a strong positive correlation between Set7/9 and TP73 showed poor survival outcome compared with the cohort where Set7/9 and TP73 exhibited a strong negative correlation (Figure 5). Similar results were obtained for breast cancer patients (Figure 5; Supplementary Data). Collectively, these results suggest that disregulation of Set7/9 activity, which is manifested by the loss of TP73 repression, in the tumours where E2F1 has an oncogenic role,that is, lung and breast tumours, negatively correlates with the survival of patients.

Both E2F1 and Set7/9 are necessary for efficient activation of cyclin E transcription. On the basis of our *in silico* results, we screened seven lung cancer cell lines with different p53 statuses for the levels of Set7/9, E2F1, and cyclin E expression (Supplementary Data; Supplementary Table 1). These data were then correlated with cell cycle and apoptotic response to DNA damage in the cell lines examined (Figure 6). Collectively, the results of experiments presented in Figure 6 suggest that the levels of cyclin E expression depended on the presence of both Set7/9 and E2F1 but were not affected by p53 (Figure 6a). For example, low expression of Set7/9 in H520 and H1650 cells correlated with very low levels of expression for cyclin E, irrespective of the E2F1 status. Yet, H460 cells expressed normal level of Set7/9, but an attenuated level of E2F1. As a result, these cells poorly expressed cyclin E. Moreover, the level of cyclin E expression correlated strongly with the cell-cycle profiles of the cell lines examined (Figure 6b). Specifically, those cell lines that expressed low levels of cyclin E underwent cell-cycle arrest preferentially in G1/S phase and the ones that expressed high levels of cyclin E were arrested in G2/M after DNA damage.

To confirm that the correlations between Set7/9, E2F1 and cyclin E expression are meaningful in respect to cell-cycle progression, we transiently knocked down E2F1 expression in H522 cells (high expression of E2F1 and Set7/9) and measured the level of cyclin E expression by western blotting before and after DNA damage (Figure 6c). The attenuation of E2F1 expression resulted in a significant decrease in cyclin E. Importantly, levels of Set7/9 did not change upon siRNA-E2F1 treatment. Collectively, these data establish a functional link between the expression of cyclin E, E2F1, and Set7/9.

Furthermore, the ability of these cells to undergo apoptosis solely depended on the presence of E2F1 and wild-type p53,

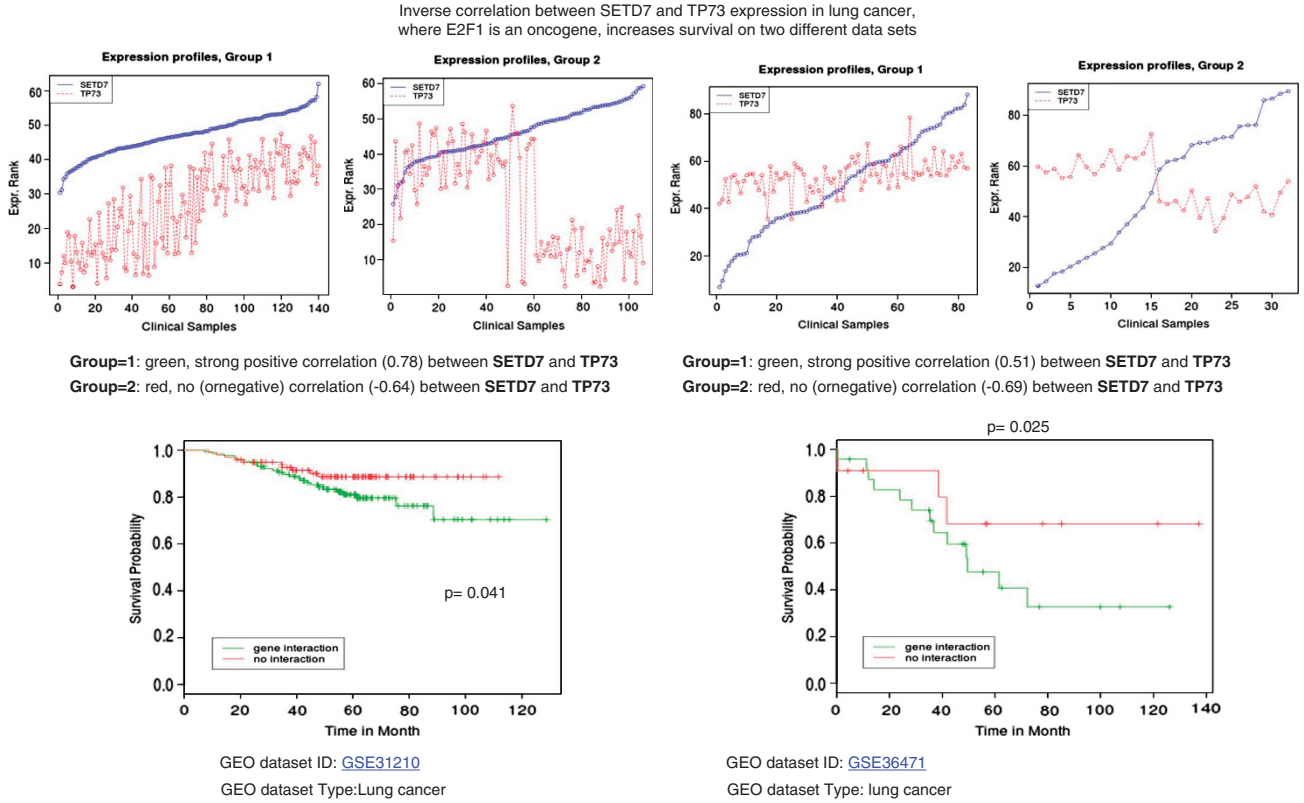

Figure 5 Inverse correlation between Set7/9 and TP73 expression, as judged from two lung cancer data sets (GSE31210, left and GSE36471, right), increases survival of patients. A statistical procedure was implemented to divide the patients with lung cancer into two cohorts. The first one (Expression profiles, Group 1) was enriched with the positive correlation between expression levels of Set7/9 (blue dots) and TP73 (red dots). The second group of patients with negative correlation (Expression Profiles, Group 2) formed the second cohort. The number of clinical samples for each group is indicated below. To identify statistical differences in survival outcome between these two groups (lower panel), the R statistical package was used to perform statistical tests and to derive the *P*-value (shown inside). The Y axis denotes relative survival probability. The X axis denotes the time of survival in months. Vertical bars on the graph curves indicate the survival measurements. A strong positive correlation between Set7/9 and TP73 (green line) shows poor survival outcome compared with the negative correlation between these two genes (red line)

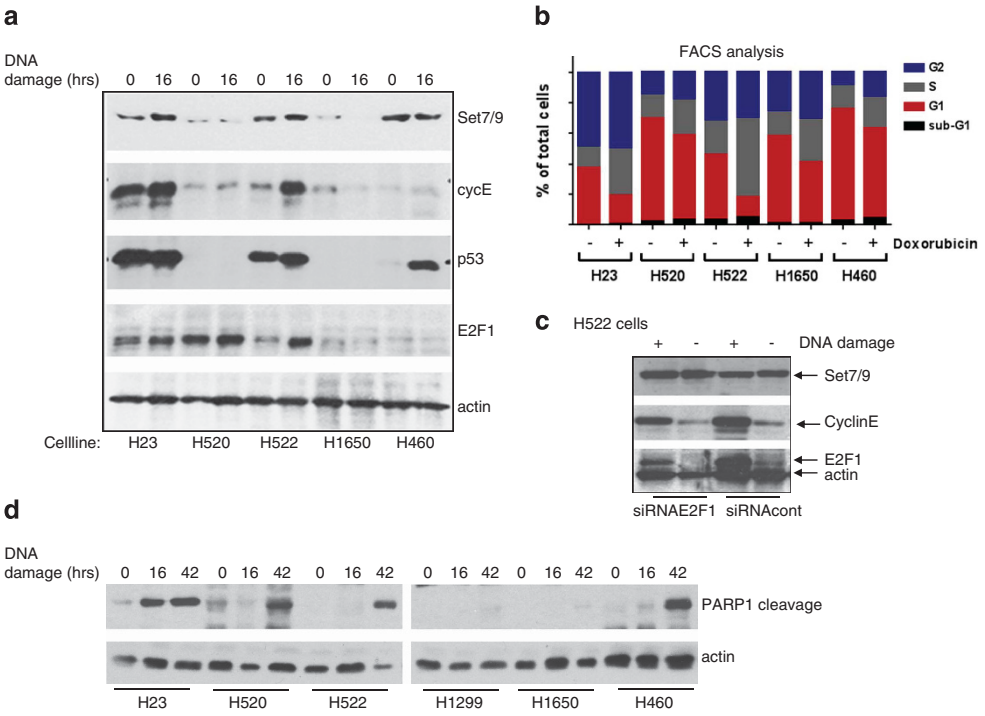

Figure 6 Analysis of different lung tumour cell lines reveals variable expression of Set7/9, E2F1, and cyclin E. (**a**) The panel of indicated lung tumour cell lines were treated with doxorubicin for the indicated periods of time and analysed for Set7/9, cyclin E, p53, and E2F1 levels. Actin was used as a loading control. (**b**) The same cell lines treated similarly as in (**a**) were also analysed by FACS for cell-cycle distribution. (**c**) H522 cells were treated with E2F1-specific or scramble siRNA followed by doxorubicin treatment to induce DNA damage. Treated cells were assayed for the levels of Set7/9, cyclin E, and E2F1 protein expression. Actin signal was used for normalisation. (**d**) The cell lines used in A were treated for various times with doxorubicin and analysed by western for the presence of the PARP cleavage product (marker of apoptosis)

but not on the presence of Set7/9 (Figure 6d, compare H23, H520, H522, and H460), reiterating our results on Set7/9-mediated repression of *TP73* expression (Figure 3g). Thus, our results strongly argue that the Set7/9-E2F1 axis, by driving the expression of cyclin E, has an important role in cell-cycle regulation of lung cancer cells upon DNA damage.

Discussion

Lysine methylation of histones has an important role in cell-cycle progression and DDR.[1,56] However, recently published reports suggest that methylation of non-histone targets is also instrumental in these processes. Several KMTases, besides Set7/9, were found to methylate important cell-cycle regulators (Rb,[57] p53,[58,59] heat shock protein 90 (HSP90)[60,61]). In this study, we show that Set7/9 has a critical role in regulation of cell cycle by promoting E2F1-dependent transcription of the *CCNE1* gene upon DNA damage. Previously, we have reported that Set7/9 affected cell cycle in a p53-dependent manner.[6] Thus, we hypothesised that the loss of Set7/9 would result in G2/M arrest. However, despite the lower level of p21 expression in Set7/9KD cells *versus* control cells (data not shown and Ivanov *et al*.[6]), we observed a profound arrest of Set7/9$^-$ cells in G1/S. This effect was due to attenuation of cyclin E expression. Importantly, we found that this phenomenon was p53 independent, but E2F1 dependent, and was reproducible upon treatment with different forms of DNA damage, including doxorubicin, gamma- and UV-irradiation.

How is E2F1 activity regulated by Set7/9? Previously, methylation of E2F1 by Set7/9 was reported to attenuate its protein stability *via* increased poly-ubiquitylation and subsequent degradation by 26S proteasome.[17] An apparently contradictory report from Xie *et al.*[62] suggests that Set7/9 stabilises and activates the E2F1 transcription factor in response to DNA damage. Our data show that, in agreement with previously reported data,[17] Set7/9 indeed attenuates the level of E2F1 expression. However, Set7/9 is required for the transcriptional activity of E2F1, thus forming a regulatory loop (Figure 7). This type of negative feedback regulation is widespread in nature. For example, yeast activator Gal4 needs to by ubiquitinylated for its full transcriptional activity, which subsequently causes its degradation.[63]

Interestingly, we found that Set7/9 inversely regulates the E2F1 target genes, *CCNE1* and *TP73* (Figures 3 and 4). These results indicate that Set7/9 is likely situated upstream of E2F1 and coordinates the final transcriptional outcome. Future studies should shed light on the molecular mechanisms of this regulation.

Our findings on the Set7/9-E2F1-cyclin E axis have important ramifications for general understanding of the molecular mechanisms of tumourigenesis, bearing in mind that E2F1-dependent overexpression of cyclin E is considered to be a 'classical' oncogenic insult causing DDR as an anti-oncogenic barrier.[64] The fact that the genetic abrogation of Set7/9 in non-transformed cells[7,8] does not affect their cell cycle or survival, makes Set7/9 a very attractive

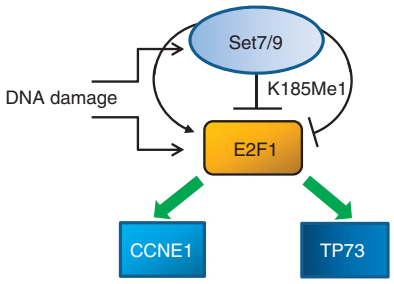

Figure 7 Set7/9 differentially regulates E2F1-dependent genes in response to DNA damage. A model depicts the effect of Set7/9 on E2F1 activity. On the one hand, Set7/9 co-activates E2F1-dependent transcription of *CCNE1* gene (left). On the other hand, Set7/9 represses E2F1-dependent transcription of Tp73 (right). In addition, Set7/9-mediated methylation of E2F1 on k185 results in de-stabilisation of the latter

pharmacological target in tumours that are critically dependent on cyclin E levels.[65]

Materials and Methods

Cell line manipulations. All cells used in this study were purchased from ATCC (Manassas, VA, USA), if not stated otherwise. Human lung adenocarcinoma cell lines H1299, H23, H520, H522, H1650, and H460 were propagated in Roswell Park Memorial Institute (RPMI) medium (Gibco, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS) (Gibco). Human osteosarcoma cell line U2-OS and its derivatives as well as human embryonic kidney cell line HEK-293 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% FBS (Gibco). Cells were grown at 37°C in a humidified atmosphere with 5% CO_2. U2-OS cells with stable expression of shRNA against Set7/9 and p53 were obtained by lentiviral infection as described previously[16] (Lezina CDDs, 2013). U2-OS cells with inducible expression of shRNA against Set7/9 and the reference cell line were generated from the commercially available U2-OS Tet-On cells (Clontech/Takara Bio Company, Mountain View, CA, USA) stably transfected with pSuperior or pSuperior-shRNA-Set7/9, respectively. Details of the cloning are available upon request.

E2F1 knockdown by specific siRNA. Downregulation of E2F1 expression was achieved by transfecting cells with specific siRNA against E2F1 as previously described.[17,66] Scrambled siRNA against luciferase served as a negative control. Cells were seeded on 60 mm dishes and were transfected the following day with 100 nM of the corresponding si-RNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were harvested 24 h after transfection.

Cdk2 and Cdk4 kinase activity. $5 \times 10E6$ of U2-OS parental control cells and cells with knockdown expression of Set7/9 and p53 were treated with doxorubicin for the indicated times and subject to lysis in radioimmunoprecipitation assay (RIPA) buffer. The resulting extracts were incubated overnight at $+4$°C with 1 μg of anti-cdk2 or anti-cdk4 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in the presence of phosphatase inhibitors (Roche, Basel, Switzerland). Following morning the immunoprecipitated complexes were recovered by adding 20 μl of protein A beads (Repligen, Waltham, MA, USA). Following washes with RIPA buffer, the cdk2/cyclin E and cdk4/cyclin D complexes were incubated in the presence of γ-[P^{32}]-ATP and the recombinant histone H1 protein for 30 min at room temperature. The radioactively labelled phosphorylated H1 proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), dried, and exposed with film.

RNA isolation and relative quantification RT-PCR. Total RNA was extracted from the cultured cells using the TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. For relative quantification RT-PCR analysis of *p21*, *CCNE1, TP73*, and β-actin mRNA, 1 μg of total RNA was reverse transcribed to cDNA with oligod(T) using Ready-to-Go kit (GE Healthcare, Amersham, UK). Then real-time PCR was performed on an RotorGene 6000 PCR machine (Qiagene, Hilden, Germany) using SYBR green dye (Invitrogen). The 20-μl PCR reaction included 1 μl RT product, $1 \times$ QuantiTect SYBR green PCR Master Mix, and 0.5 μM each sense and antisense primers. The reactions were incubated in a 96-well plate at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. All reactions were run in triplicate. After the reactions, the C_T values were determined using fixed threshold settings. Relative amounts of *p21*, *CCNE1*, and *TP73* mRNAs were normalised to β-actin mRNA. Expression of these genes was analysed by RT-PCR using the following primers: (p21) 5-gacaccactggagggtgact, 3-ctcttggagaagatcagccg; (CCNE1) 5-caaactcaacgtg caagcctc, 3-gcccagctcagtacaggcag; (TP73) 5-cacgtttgagcacctctgga, and 3-gaactg ggccatgacagatg. Primers to β-actin were purchased from PrimerDesign, (Southampton, UK).

ChIP assay. ChIP assays were performed as described previously.[46] Briefly, 3×10^6 cells per sample were crosslinked with 1% formaldehyde for 15 min. Crosslinking was neutralised with 0.125 M glycine, and cells were scraped in phosphate buffer saline (PBS). Chromatin was sonicated using the Diagenode (Liège, Belgium) Bioruptor for 15 min with 30-s pulse/pause cycles in polycarbonate tubes on ice to shear chromatin to 300- to 600-bp fragments. Unsheared debris was spun down, and then the chromatin was incubated overnight with the appropriate antisera, concurrent with the blocking of protein G-Sepharose beads using 2.5% bovine serum albumin (BSA). Immune complexes were then precipitated using 'blocked' protein G beads for 4 h at 4°C, washed three times, and then eluted. Immunoprecipitated DNA was purified and quantitative PCR was performed with 1 μl of DNA to assess E2F1 binding and modified histone levels. The following antibodies were used: antibodies for CHIP: HDAC2 (Millipore 05-814, Billerica, MA, USA), anti-E2F-1 (clones KH20 and KH95, Millipore 05-379), anti-Histone H3-K4me1 (Abcam ab8895, Cambridge, UK), anti-acetyl-Histone H3 (Millipore 06-599), anti-acetyl-Histone H4 (Millipore 06-866), and anti-H3K9me3 (Diagenode C15410056). To examine the binding of E2F1 and histone modifications of *CCNE1* and *TP73* the following primers were used: cycE -0.4 Kb up start site (in close proximity of E2F1 biding site): F-ACACATCCCCTTGGCTCA; R-CGGGTGGAATGTAAACACG; TP73 -1 Kb upstream of the start site: F-TGAGCCATGAAGATGTGCGAG; R-GCTGCTTATGG TCTGATGCTTATG.

Western blotting. p53, E2F1, Cdk2, Cyclin E, Cdk4, cyclin D and Wee1 protein levels were quantified by western blot analysis of whole-cell extracts using antibodies against the corresponding proteins. These samples were normalised by blotting with an antibody against α-tubulin. The following antibodies were used: Anti-p53 (Ab-6, Oncogene, San Diego, CA, USA), anti-Cyclin E (sc-15254, Santa Cruz Biotechnology), anti-E2F1 (Millipore), Cdk2, cyclin D, Cdk4 (Santa Cruz Biotechnology), and anti-α-actin (B-7, Santa Cruz Biotechnology).

Luciferase assay of 3-UTR constructs. The luciferase reporter plasmids with wild-type CCNE1 promoter and its E2F1 binding-deficient variants were kindly provided by JR Nevins laboratory. For luciferase reporter assays, cells were cultured in 6-well plates, and each well was transfected with 0.5 μg of luciferase reporter plasmid, 0.5 μg of β-galactosidase expression plasmid (Ambion, Life Technologies, Carlsbad, CA, USA), and equal amounts of scrambled negative control RNA, pre-miR-26a, pre-miR-16, or anti-miR-16 using Lipofectamine 2000 (Invitrogen). The β-galactosidase plasmid was used as a transfection control. At 24 h post transfection, cells were assayed using luciferase assay kits (Promega, Madison, WI, USA). The data depicted are representative of three independent experiments performed on different days.

Cell-cycle analysis and BrdU staining. Cells were harvested, washed once with PBS, and fixed in 70% ethanol overnight. Staining for DNA content was performed with 50 μg/ml propidium iodide and 1 μg/ml RNase A for 30 min. Analysis was performed on a FACS Canto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with the Cell Quest Pro software. Cell-cycle modelling was performed with the Modfit 3.0 software (Verity Software House, Topsham, ME, USA).

Staining of the cells with BrdU was carried out with the FITS BrdU Flow kit according to the manufacturer's instructions (BD Pharmingen, Franklin Lakes, NJ, USA). Briefly, $5 \times 10E5$ U2-OS and U2-OS Set7/9KD cells were non-treated or pulse-treated with 0.1 μM of doxorubicin at the indicated time points, washed with PBS with subsequent labelling with 10 μM BrdU. Following the incubation, cells were fixed with 0.5% paraformaldehyde, permeabilised, treated with DNAse I,

incubated with anti-BrdU-FITS conjugated antibody and stained with 7-AAD to visualise DNA. The resulting samples were analysed by fluorescence-activated cell sorting (FACS).

Statistical analysis. All presented images of western blotting and cell-cycle assay are representative of at least three independent experiments. Relative quantification RT-PCR, luciferase reporter, and cell viability assays were performed in triplicate, and each experiment was repeated three to five times. The data shown are the mean ± S.D. of at least three independent experiments. Statistical significance was considered at $P < 0.05$ using the Student's *t*-test.

Immunofluorescence microscopy. Mitotic U2-OS or U2-OS Set7/9KD cells were detected by staining with anti-phospho-Histone H3 (Ser10) antibodies conjugated with Alexa 647 (Cell Signaling, Danvers, MA, USA; #9716). The nuclei were visualised with Hoechst 33342 dye (Sigma, St. Louis, MO, USA; 14533). Cell plates were analysed using an automated Operetta Imaging System (Perkin-Elmer, Waltham, MA, USA) at × 20 magnification with the Harmony 3.1 software (Perkin-Elmer). To calculate main readout values (number of cells, fraction of cells in M-phase) RMS Cell Cycle Classification application (Perkin-Elmer) was used. The 23 independent fields were analysed in all wells of four independent replicates. The percentage of mitotic cells was calculated as a ratio of all M-phase positive cells to all cells in the well.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Cell Death and Differentiation website (http://www.nature.com/cdd)