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Che-1 Promotes Tumor Cell Survival by Sustaining Mutant p53 Transcription and Inhibiting DNA Damage Response Activation

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SUMMARY

Che-1 is a RNA polymerase II binding protein involved in the regulation of gene transcription and, in response to DNA damage, promotes p53 transcription. In this study, we investigated whether Che-1 regulates mutant p53 expression. We found that Che-1 is required for sustaining mutant p53 expression in several cancer cell lines, and that Che-1 depletion by siRNA induces apoptosis both in vitro and in vivo. Notably, loss of Che-1 activates DNA damage checkpoint response and induces transactivation of p73. Therefore, these findings underline the important role that Che-1 has in survival of cells expressing mutant p53.

INTRODUCTION

Che-1 (also named AATF and Traube) is an evolutionary conserved RNA polymerase II binding protein involved in the regulation of gene transcription (Fanciulli et al., 2000; Thomas et al., 2000; Lindfors et al., 2000; Page et al., 1999; Burgdorf et al., 2004; Bruno et al., 2002). Che-1 interacts with Rb and interferes with the Rb-mediated recruitment of histone deacetylase I on the promoters of E2F1-responsive genes, inhibiting the Rb growth suppressing functions (Fanciulli et al., 2000; Bruno et al., 2002). In agreement, the mouse Traube is essential for proliferation in early embryogenesis (Thomas et al., 2000). In addition to pro-proliferative function, Che-1 exhibits strong anti-apoptotic activity (Page et al., 1999; Guo and Xie, 2004), and this protein is downregulated during apoptosis through its interaction with MDM2 (De Nicola et al., 2007) and NRAGE (Di Certo et al., 2007). Recently, we have shown that in response to DNA damage, Che-1 is localized to the *p53* promoter, increasing transcription of this gene and contributing to the increase in p53 protein levels after DNA damage (Bruno et al., 2006).

Mutant p53 proteins (mtp53) are abundantly present in cancer cells of various histotypes (Soussi, 2000), where they show oncogenic properties (Vousden and Lu, 2002). One common mechanism underlying this "gain of function" is through protein-protein interaction between mtp53 and other cellular proteins, leading to the disruption of the normal functions of these molecules. Indeed, some mtp53 proteins can interact with and inhibit the transcriptionally active forms of the p53 homologs p73 and p63, leading to reduced apoptotic response and chemoresistance (Li and Prives, 2007). More recently, it has

Significance

The p53 gene is the most frequent target for genetic alterations in human cancer. Mutant p53 proteins not only lose wild-type p53 tumor suppressor activity, but gain specific properties contributing to tumor aggressiveness and chemoresistance and are often correlated with poor prognosis. Therefore, mutant p53 is considered a target for specific anticancer treatments. Here, we provide evidence that Che-1 depletion strongly decreases mutant p53 expression in human cancer cells. In addition, Che-1 depletion induces p73 transcription and apoptosis by activating DNA damage checkpoint in these cells. These findings suggest a therapeutic approach involving the simultaneous modulation of p73 and mutant p53 levels. This approach could be used to target the large fraction of human tumors harboring p53 mutations.

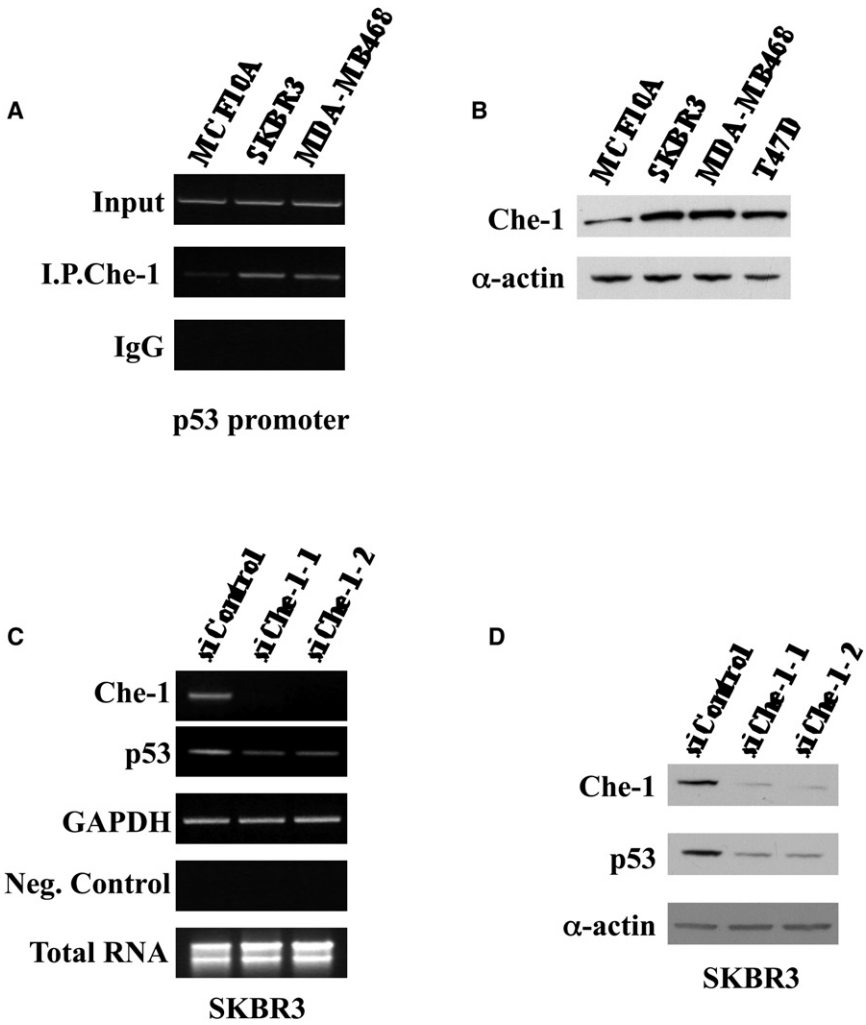

Figure 1. Che-1 Regulates Mutant p53 Expression

(A) Total cell extracts (TCEs) from the indicated cell lines were subjected to chromatin immunoprecipitation (ChIP) with anti-Che-1 antibody (Ab). Immunoprecipitates from each sample were analyzed by PCR and a sample representing linear amplification (0.2–0.4 μl) of the total input chromatin (input) was included in the PCRs as a control. Additional control included a precipitation performed with nonspecific IgGs.

(B) Western blot (WB) analysis of TCEs from the indicated cell lines.

(C and D) SKBR3 cells were transiently transfected with siRNA GFP (siControl) or two different siRNA Che-1 (siChe-1-1 and siChe-1-2) and 24 hr later total RNA and proteins were extracted. (C) RT-PCR analysis of the indicated genes. (D) WB with the indicated Abs. See also Table S1.

been demonstrated that some mtp53 physically interact with Mre11, a protein involved in DNA damage response, generating genetic instability by ATM pathway inactivation (Song et al., 2007). Finally, although most of mtp53 do not directly bind DNA, several findings indicate that these proteins can exert their oncogenic activity by an aberrant transcriptional activity (Strano et al., 2007).

Mtp53 molecules are characterized by a prolonged half-life compared with that of the wt-p53 protein and by the inability to recognize specific DNA binding sites (Sigal and Rotter, 2000). The molecular basis of the extended half-life of mtp53 proteins might depend on the inefficient degradation exerted by MDM2, a direct transcriptional target of wt-p53 (Haupt et al., 1997), but also on the fact that in many tumors mtp53 might be stabilized by DNA damage response pathways (Bartkova et al., 2005; Gorgoulis et al., 2005).

Taken together, these observations suggest mtp53 as a target for specific anticancer treatments. Indeed, a number of strategies for targeting mtp53 have been designed, for example peptides and small molecules that restore active conformation and DNA binding to mtp53 (Selivanova and Wiman, 2007), or disrupt the protein complex mtp53/p73 (Di Agostino et al., 2008). In addition, depletion of mtp53 by RNA interference renders tumor cells more sensitive to anticancer drugs and reduces tumor malignancy in vitro and in vivo (Bossi et al., 2006; Bossi et al., 2008). Thus, we asked whether Che-1 also regulates mtp53 expression, and whether Che-1 inhibition could affect proliferation of cancer cells carrying mtp53.

RESULTS

Che-1 Regulates Mutant p53 Expression

In response to DNA damage Che-1 is recruited onto the *p53* promoter, and in such a way contributes to an increase of wt-p53 expression (Bruno et al., 2006). Thus, we hypothesized that mtp53 expression might also be regulated by Che-1. To test this possibility, we first investigated the presence of Che-1 onto the *p53* promoter by performing chromatin immunoprecipitations (ChIP) in cancer cell lines carrying different mutants of p53 (Table S1 available online) and analyzing the NF-κB binding region of the *TP53* promoter (Bruno et al., 2006). As shown in Figure 1A, ChIP analysis revealed Che-1 physically associated with the *p53* promoter in SKBR3 and MDA-MB468 cells, whereas it was barely detectable in primary breast epithelial MCF10A cells. In addition, Che-1 protein was found accumulated in cancer cells with respect to levels observed in normal MCF10A cells (Figure 1B). Next, we tested the effects of Che-1 depletion on mtp53 expression. For this purpose SKBR3 cells were transfected with two independent siRNA duplexes specific for Che-1 sequences or with siRNA oligos specific for the green florescent protein (GFP) as negative control. Efficient depletion of Che-1 strongly decreased mtp53 expression both at the level of mRNA (Figure 1C) and at the level of protein (Figure 1D) when compared to control siRNA, and similar results were obtained depleting Che-1 expression by another specific siRNA duplex (not shown). Taken together, these results show that in cancer

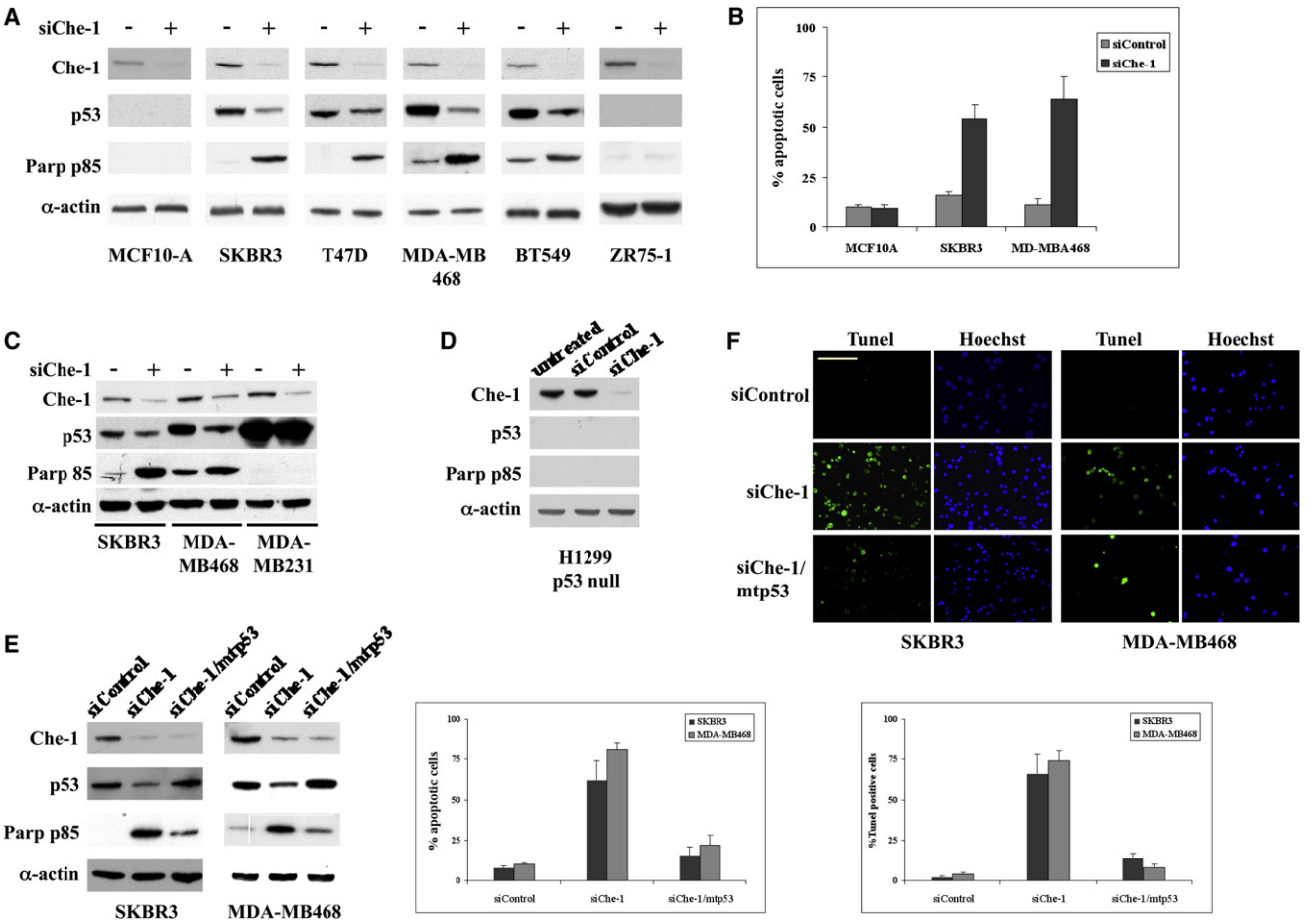

Figure 2. Che-1 Depletion Induces Apoptosis in Cells Expressing mtp53

(A) Indicated cell lines were transiently transfected with siRNA GFP (−) or siRNA Che-1 (+) and 24 hr later TCEs were analyzed by WB with the indicated Abs.

(B) MCF10A, SKBR3, and MDA-MB468 cells were transfected as in (A). Twenty-four hours later, cell death was assayed by trypan blue staining, and percentages represent trypan blue incorporating cells. Data are presented as the mean ± SD from three independent experiments performed in duplicate.

(C and D) TCEs from the indicated cell lines transiently transfected as in (A), were subjected to WB with the indicated Abs.

(E) SKBR3 and MDA-MB468 cells were transiently transfected with siRNA GFP (siControl), siRNA Che-1 (siChe-1), or siChe-1 and mtp53 R175H (SKBR3) or R273H (MDA-MB468) expression vectors. WB analysis of TCEs with the indicated Abs is shown on the left. Cell death analysis performed by trypan blue staining as in (B) is shown on the right. Data are presented as the mean ± SD from three independent experiments performed in duplicate.

(F) TUNEL assay of SKBR3 and MDA-MB468 cells transfected as in (E). Shown on the top are representative fields of TUNEL-positive nuclei. The scale bar represents 100 μm. As shown on the bottom, TUNEL positivity is presented as the percentage of positive nuclei to total cell number. The data represent the mean ± SD from three independent experiments.

cells carrying mtp53, Che-1 is present onto the *p53* promoter and is required for its expression.

Che-1 Depletion Induces Apoptosis in Cells Expressing mtp53

To validate the results observed in SKBR3 cells, we tested the effect of Che-1 depletion by siRNA in other cancer cell lines expressing different mtp53 (Table S1). As shown in Figure 2A, Che-1 inhibition strongly decreased mtp53 protein levels in these cells, confirming that Che-1 regulates the expression of this gene. Strikingly, Che-1 depletion induced extensive cell death in all cell lines expressing mtp53 (Figures 2A and 2B), and cell death was accompanied by cleavage of the poly(ADP-ribosylating) enzyme PARP-1, a specific hallmark of apoptosis (Figure 2A). In contrast, depletion of Che-1 in MCF10A cells did not induce PARP-1 cleavage, or cause cell death (Figures 2A and 2B). Notably, ZR75-1, a cancer cell line expressing wt-p53, did not show apoptosis when Che-1 expression was inhibited (Figure 2A), and similar effects were observed in MDA-MB231, a cancer cell line carrying mtp53, but in which Che-1 depletion did not affect p53 levels, probably because of its elevated stabilization in these cells (Figure 2C). Consistent with these results, Che-1 downregulation did not induce PARP-1 cleavage in $p53^{-/-}$ H1299 cells (Figure 2D). To test whether Che-1 depletion induces apoptosis by downregulating mtp53, we depleted Che-1 in SKBR3 and MDA-MB468 cells in absence or in presence of specific mtp53 overexpression (see Table S1). As shown in Figures 2E and 2F, ectopic mtp53 expression rescued apoptosis induced in Che-1 depleted cells. In addition, HT29, a colon cancer cell line expressing R273H mtp53, exhibited p53

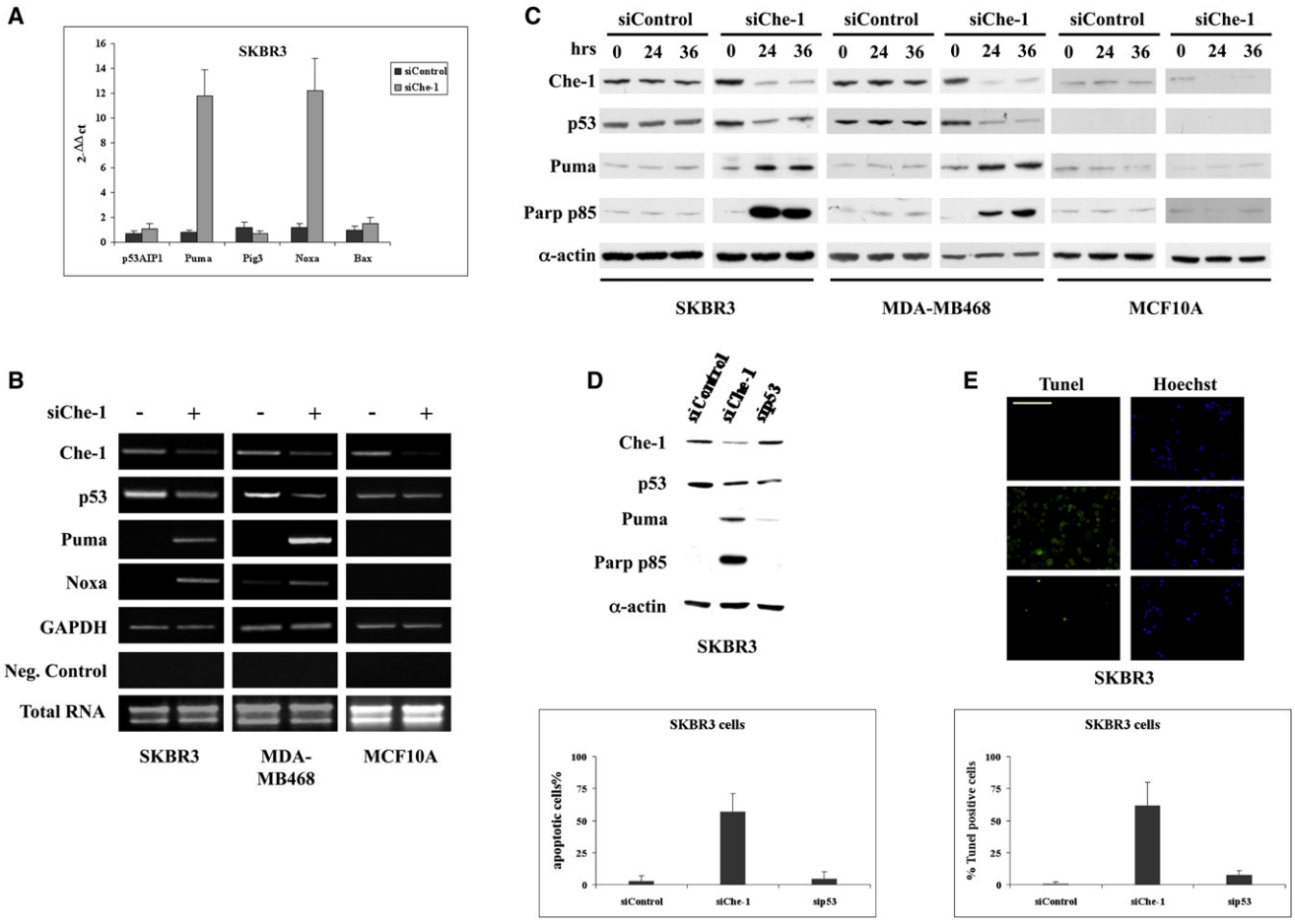

Figure 3. Che-1 Depletion Induces Puma and Noxa Expression

(A) Real-time QRT-PCR for candidate pro-apoptotic genes was performed after transient transfection of SKBR3 cells with siRNA GFP (siControl) or siRNA Che-1 (siChe-1). Values were normalized to GAPDH expression. Error bars represent the standard error of three independent experiments.

(B) Equal amounts of RNA (RNA input) from the indicated cell lines transfected as in (A) were analyzed by RT-PCR (25–30 cycles) for the expression of the indicated genes. The negative control lanes represent RT-PCR in the absence of cDNA.

(C) MCF10A, SKBR3, and MDA-MB468 cells were transiently transfected as in (A) and after 24 hr or 36 hr TCEs were analyzed by WB with the indicated Abs.

(D) SKBR3 cells were transiently transfected with siRNA GFP (siControl), siRNA Che-1 (siChe-1), or siRNA p53 (sip53). WB analysis of TCEs with the indicated Abs is shown on the top. Cell death analysis assayed by trypan blue staining is shown on the bottom. Percentages represent trypan blue incorporating cells. Data are presented as the mean ± SD from three independent experiments performed in duplicate.

(E) TUNEL assay of SKBR3 cells transfected as in (D). Representative fields of TUNEL-positive nuclei are shown on the top. The scale bar represents 100 μm. As shown on the bottom, TUNEL positivity is presented as the percentage of positive nuclei to total cell number. The data represent the mean ± SD from three independent experiments. See also Figure S1.

decrease and cell death in response to Che-1 depletion, but apoptosis was counteracted by mtp53 overexpression (not shown). Altogether, these results show that Che-1 inhibition induces apoptosis in cancer cells by suppressing mtp53 expression.

Che-1 Depletion Induces Puma and Noxa Expression

Next, we investigated the mechanism/s by which Che-1 depletion induces apoptosis. We initially assayed by real-time quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) (the expression of well-characterized apoptotic genes (*Bax*, *Noxa*, *p53AIP1*, *Puma*, and *Pig3*) in SKBR3 cells after Che-1 depletion. From this analysis, it was observed that *Puma* and *Noxa*, but no other proapoptotic genes, were induced by Che-1 depletion (Figure 3A). Accordingly, Che-1 depletion activated *Puma* promoter in SKBR3 and in MDA-MB468 cells, whereas no induction was observed in MCF10A or ZR75-1 cells (Figure S1A). In contrast, no induction of the *Pig3* promoter was found in any cell line analyzed (Figure S1B). Puma and Noxa activation in response to Che-1 depletion was further confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) in SKBR3 and in MDA-MB468 cells (Figure 3B), and in these cells, unlike in MCF10A cells, the increase of Puma protein within 24 hr corresponded with PARP-1 cleavage (Figure 3C). Strikingly, specific mtp53 depletion in SKBR3 cells by siRNA did not produce Puma activation, PARP-1 cleavage (Figure 3D) or

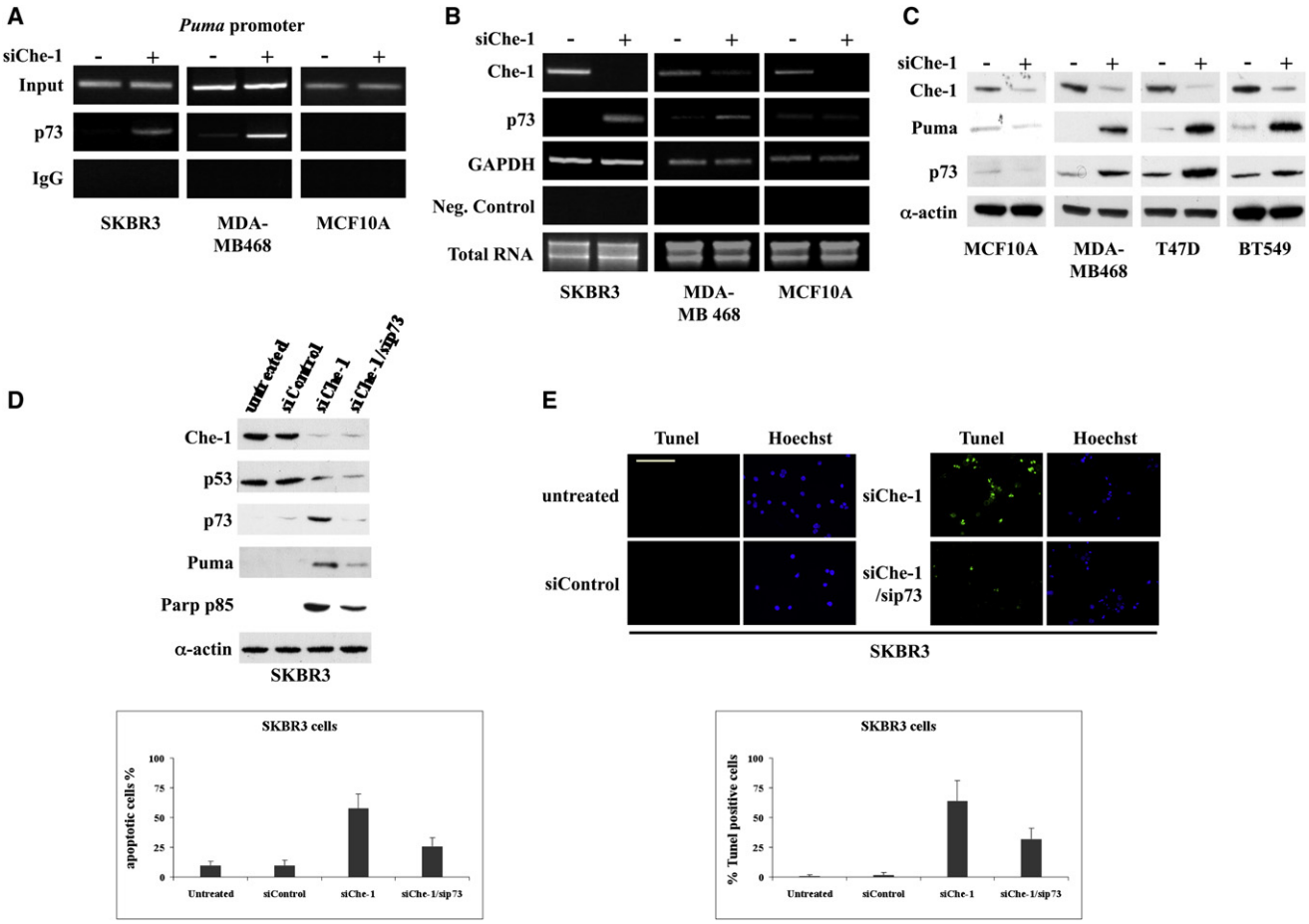

Figure 4. Che-1 Inhibition Activates p73 Expression

(A) Indicated cells were transiently transfected with siRNA GFP (−) or siRNA Che-1 (+). Cells were then subjected to ChIP with anti-p73 Ab or control IgGs.

(B) Equal amounts of RNA (RNA input) from indicated cells transfected as in (A) were analyzed by RT-PCR (25–30 cycles) for the expression of the indicated genes.

(C) TCEs from indicated cells transiently transfected as in (A) were analyzed by WB with the indicated Abs.

(D) SKBR3 cells were transiently transfected with siRNA GFP (siControl), siRNA Che-1 (siChe-1), or siChe-1 and siRNA p73 (sip73). WB analysis of TCEs with the indicated Abs is shown on the top. Cell death analysis assayed by trypan blue staining is shown on the bottom. Percentages represent trypan blue incorporating cells. Data are presented as the mean ± SD from three independent experiments performed in duplicate.

(E) TUNEL assay of SKBR3 cells transfected as in (D). Representative fields of TUNEL-positive nuclei are shown on the top. The scale bar represents 100 μm. As shown on the bottom, TUNEL positivity is presented as the percentage of positive nuclei to total cell number. The data represent the mean ± SD from three independent experiments. See also Figure S2.

apoptosis (Figures 3D and 3E), suggesting that mtp53 depletion is required but not sufficient for apoptosis induction. Thus, specific Che-1 depletion in mtp53 expressing cells leads to induction of proapototic effector genes, PARP-1 cleavage, and cell death.

Che-1 Depletion Activates p73 Expression

Several findings support the hypothesis that mtp53 promotes pro-oncogenic activities by sequestering and inactivating the p53 homologs p63 and p73 (Blandino et al., 1999; Di Como et al., 1999; Gaiddon et al., 2001; Strano et al., 2002; Li and Prives, 2007). Given that *Puma* is a specific p73 target gene (Ramadan et al., 2005; Melino et al., 2004), we hypothesized that Che-1 depletion might promote apoptosis through activation of p73. We first tested on Che-1 depletion, the presence of p73 onto the *Puma* promoter by ChIP analysis in SKBR3, MDA-MB468 and MCF10A cells. As is shown in Figure 4A, Che-1 depletion produced a strong recruitment of p73 onto the p53 family binding site of the *Puma* promoter in SKBR3 and MDA-MB468 cells, whereas this effect was not observed in MCF10A cells. According to the literature, p73 accumulation onto the *Puma* promoter might depend on the decrease of mtp53 levels, that, reducing p73 sequestration, allows the restoration of p73 transcriptional effects (Li and Prives, 2007). However, we did not observe a significant increase of overexpressed p73 activity in Che-1 depleted cells (Figure S2A), suggesting an involvement of some other mechanism. Thus, we examined whether Che-1 depletion induces p73 expression. Consistent with this hypothesis, RT-PCR analysis revealed an increase of p73 mRNA levels after Che-1 depletion in either SKBR3 or MDA-MB468 cells, but not in MCF10A cells (Figure 4B). Accordingly, several cell lines carrying mtp53 exhibited

a strong increase of p73 protein level upon Che-1 depletion (Figure 4C). In addition, the use of peptides that specifically disrupt the mtp53/p73 complex (Di Agostino et al., 2008), enhanced apoptosis induced by Che-1 depletion in SKBR3 cells (Figures S2B and S2C). To test directly whether endogenous p73 is required for cell death induced by Che-1 depletion, we investigated the effects of Che-1 inhibition in cells in which p73 expression was ablated by RNAi. Remarkably, p73 depletion substantially and consistently abrogated the effects of Che-1 inhibition. Indeed, in absence of p73 little or no Puma induction, PARP-1 cleavage or apoptosis were observed (Figures 4D and 4E). Therefore, p73 is required for the apoptotic program elicited after loss of Che-1. Altogether, Che-1 inhibition induces apoptosis not only by decreasing mtp53 levels, but also by increasing p73 expression.

Che-1 Depletion Induces DNA Damage Response

Next, we investigated the mechanism/s by which Che-1 depletion increases p73 levels in cancer cells expressing mtp53. Several findings have demonstrated that in response to DNA damage, the E2F1 transcription factor is stabilized by checkpoint activation (Lin et al., 2001; Urist et al., 2004) and recruited onto the *p73* promoter (Pediconi et al., 2003; Urist et al., 2004; Irwin et al., 2000; Lissy et al., 2000; Stiewe and Putzer, 2000). Because recent studies have revealed a specific gain of function of mtp53 in inducing genetic instability by inactivating critical checkpoint activation pathways (Song et al., 2007), we evaluated whether Che-1 knockdown might induce checkpoint activation in cancer cells expressing mtp53. As shown in Figure 5A, Che-1 depletion induced a specific increase of checkpoint activation in several cancer cells expressing mtp53, evaluated by western blot analysis of phosphorylated levels of histone H2AX, ATM, and Chk2 (Rogakou et al., 1998; Pilch et al., 2003; Bakkenist and Kastan, 2003; Ahn et al., 2002). Consistent with these findings, confocal microscopy imaging analysis using antibodies for phosphorylated forms of histone H2AX and ATM indicated a massive DNA damage foci formation after Che-1 depletion (Figure S3A). Moreover, in line with the results of Song et al. (2007), Mre11 was also found recruited in DNA damage foci (Figure S3A). Of note, Che-1 inhibition did not induce histone H2AX phosphorylation in ZR75-1 or MDA-MB231 cells (not shown). This checkpoint activation was also observed in presence of Z-Vad, a caspase inhibitor (Figure S3B), thereby excluding an activation from apoptotic DNA fragmentation. Notably, Che-1 depletion induced E2F1 stabilization in MDA-MB468 cells, associated with phosphorylation of histone H2AX (Figure 5B). Consistent with these results, Che-1 inhibition strongly increased the presence of E2F1 onto the *p73* promoter in SKBR3 and MDA-MB468 cells (Figure 5C). To evaluate whether E2F1 is directly involved in p73 activation after Che-1 depletion, we tested the effect of Che-1 inhibition in SKBR3 cells depleted of E2F1 expression. Although histone H2AX phosphorylation was still observed in these cells (Figure 5D), loss of E2F1 prevented p73 and Puma activation, PARP-1 cleavage and cell death (Figure 5D and Figure S4C). Notably, mtp53 expression rescued Puma expression induced by Che-1 depletion, although in these cells DNA damage was induced and p73 expression increased (Figure 5E). In accordance with these findings, mtp53 overexpression did not allow p73 recruitment onto the *Puma* promoter and its transcription (Figures 5F and 5G). Taken together, these results indicate that Che-1 depletion in tumor cells carrying mtp53 induces p73 activation through E2F1 stabilization and checkpoint activation, but does not affect Puma expression and apoptosis if mtp53 expression is not inhibited.

Che-1 Is Involved in DNA Repair

The data described above demonstrate that Che-1 inhibition induces checkpoint activation and E2F1 stabilization in SKBR3 and MDA-MB468 cells. To shed light on the mechanism/s by which these effects are exerted, we evaluated cell cycle profiles of Che-1-depleted MDA-MB468 and MCF10A cells 12 hr after siRNA transfection, before induction of the apoptotic process (Figure 3C). As shown in Figure 6A, MDA-MB468 Che-1-knocked down cells exhibited a cell cycle profile with a strong G_2/M depletion and a concomitant S phase accumulation. In addition, Che-1 depleted cells showed a reduced bromodeoxyuridine incorporation (Figure S4A), thus suggesting a specific replication-associated damage and inhibition of S phase progression. Similar results were observed in SKBR3 cells (not shown). Given that Che-1 is a RNA Pol II-binding protein involved in gene transcription, to understand the biological relevance of Che-1 in these phenomena, we took advantage of a high-density Affimetrix microarray analysis performed using SKBR3 cells transiently transfected with control siRNA or Che-1 siRNA (Figure S4B). In agreement with Che-1 antiapoptotic activity (Passananti et al., 2007; Bruno et al., 2008), several proapoptotic genes were found to be induced by Che-1 depletion (Figure S4C). Interestingly, among the downregulated genes in Che-1-depleted cells we identified several genes involved in DNA damage response and DNA repair (Figure S4C), and consistent with these findings, Che-1 depletion strongly reduced the repair of damaged DNA (Figure S4D). In particular, we found that loss of Che-1 downregulated Bloom syndrome helicase (BLM) and RAD17, whose role in intra-S phase checkpoint is well established (Amor-Guéret, 2006; Wu, 2007; Wang et al., 2006). These microarray data were confirmed by another microarray analysis performed in Che-1 depleted MDA-MB468 cells, and by RT-PCR (Figure 6B and Figure S5E), but expression of these genes was not found modulated by Che-1 RNAi in MCF10A control cells (Figure 6C). Furthermore, western blot analysis showed a significant reduction of BLM and RAD17 protein levels in MDA-MB468, SKBR3 and TD47 cells (Figure 6C). Interestingly, in H1299 cells stably expressing mtp53, increased levels of both BLM and RAD17 were observed (Figure S4F). To evaluate whether apoptosis after Che-1 inhibition involves BLM and RAD17 downregulation, we depleted Che-1 in SKBR3 cells in absence or in presence of BLM and RAD17 overexpression. Strikingly, restoration of BLM and RAD17 protein levels counteracted DNA damage and apoptosis induced by Che-1 ablation (Figure 6D and Figure S4G), In addition, whereas BLM depletion produced DNA damage but did not induce apoptosis, a simultaneous depletion of mutant p53 and BLM expression recapitulated the effects observed when Che-1 expression is inhibited, inducing DNA damage, p73 expression and apoptosis (Figure 6E and Figure S4H). Taken together, these results strongly support an involvement of Che-1 in DNA repair and indicate that its depletion induces replication stress in cancer cells with mutated p53.

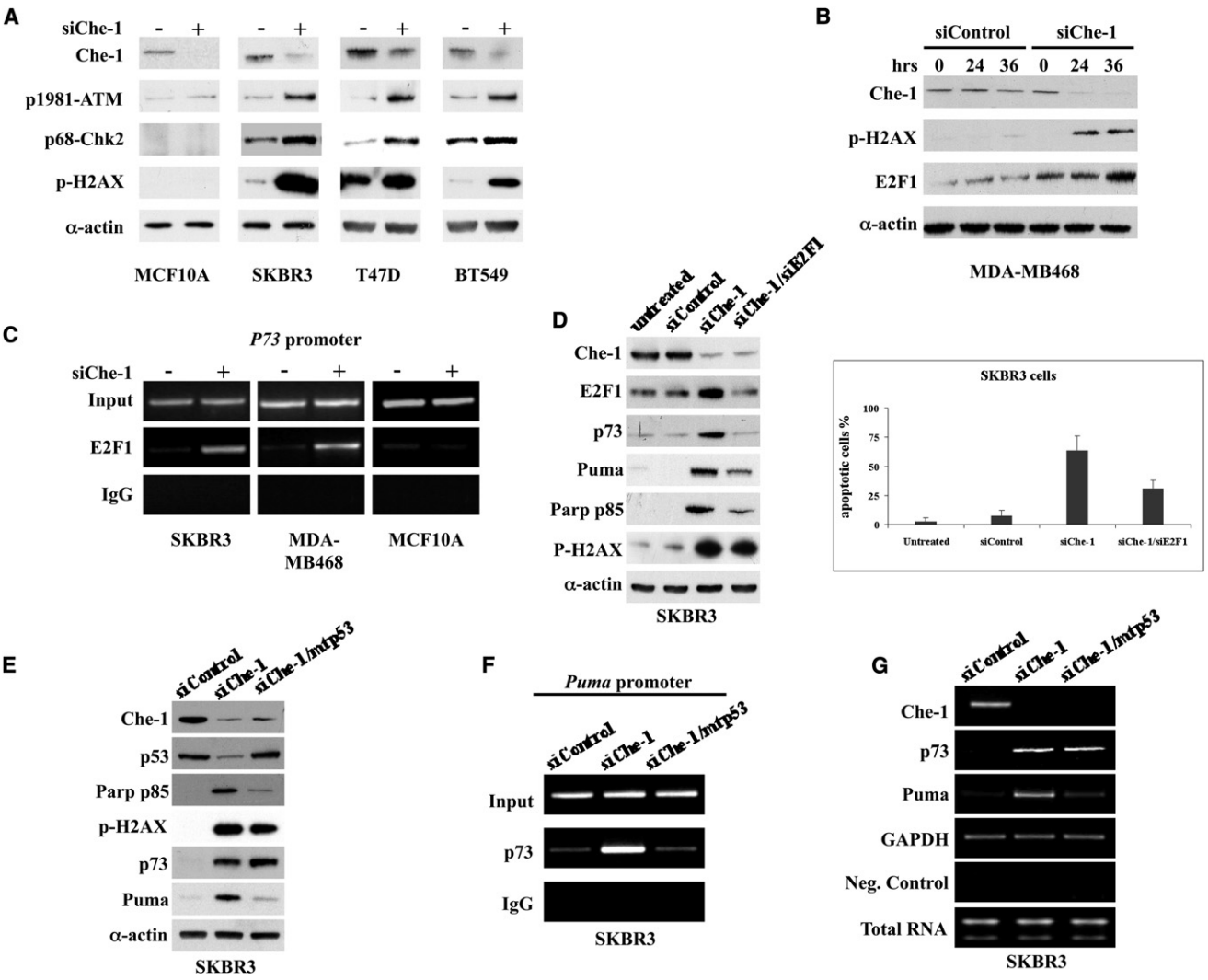

Figure 5. Che-1 Depletion Induces DNA Damage Response

(A) Indicated cells were transiently transfected with siRNA GFP (–) or siRNA Che-1 (+). TCEs were analyzed by WB with the indicated Abs.

(B) MDA-MB468 cells were transfected as in (A) and after 24 hr or 36 hr TCEs were analyzed by WB using the indicated Abs.

(C) Indicated cells were transiently transfected with siRNA GFP (–) or siRNA Che-1 (+). Cells were then subjected to ChIP using anti-E2F1 Ab or control IgGs.

(D) SKBR3 cells were transiently transfected with siRNA GFP (siControl), siRNA Che-1 (siChe-1), or siChe-1 and siRNA E2F1 (siE2F1). WB analysis of TCEs with the indicated Abs is shown on the left. Cell death analysis assayed by trypan blue staining is shown on the right. Percentages represent trypan blue incorporating cells. Data are presented as the mean ± SD from three independent experiments performed in duplicate.

(E) SKBR3 cells were transiently transfected with siRNA GFP (siControl), siRNA Che-1 (siChe-1), or siChe-1 and mtp53 R175H expression vector. WB analysis of TCEs was performed with the indicated Abs.

(F) SKBR3 cells were transfected as in (E) and subjected to ChIP with anti-p73 Ab or control IgGs.

(G) Equal amounts of RNA (RNA input) from SKBR3 cells transfected as in (E) were analyzed by RT-PCR (25–30 cycles) for the expression of the indicated genes. See also Figure S3.

Che-1 Depletion Suppresses Tumor Growth in a Mouse Xenograft Model

The results described above indicate that Che-1 is required to sustain mtp53 expression and to maintain cell survival. To further confirm these data, under more physiologic conditions, we generated an in vivo conditional RNAi model. To this aim, we produced a stable conditional depletion of Che-1 by infecting MDA-MB468 cells with either an inducible lentiviral vector carrying a specific hairpin (sh) RNA against Che-1 (MDA-MB468 ind-si/Che-1) or with a vector carrying a control hairpin (MDA-MB468 ind-si/Control) (Bossi et al., 2006). As shown in Figure 7A, depletion of Che-1 by doxycycline (Dox) treatment decreased mtp53, and increased PARP-1 cleavage. Moreover, when MDA-MB468 ind-si/Che-1 cells were treated with Dox, they exhibited DNA damage induction, with subsequent stabilization of E2F1 and activation of p73 and Puma (Figure 7B). Therefore, to perform studies in vivo, either ind-si/Che-1 or ind-si/Control engineered cells were implanted subcutaneously in *Scid/Scid* immunodepleted mice. Three weeks after injection, when injected cells generated tumor nodules

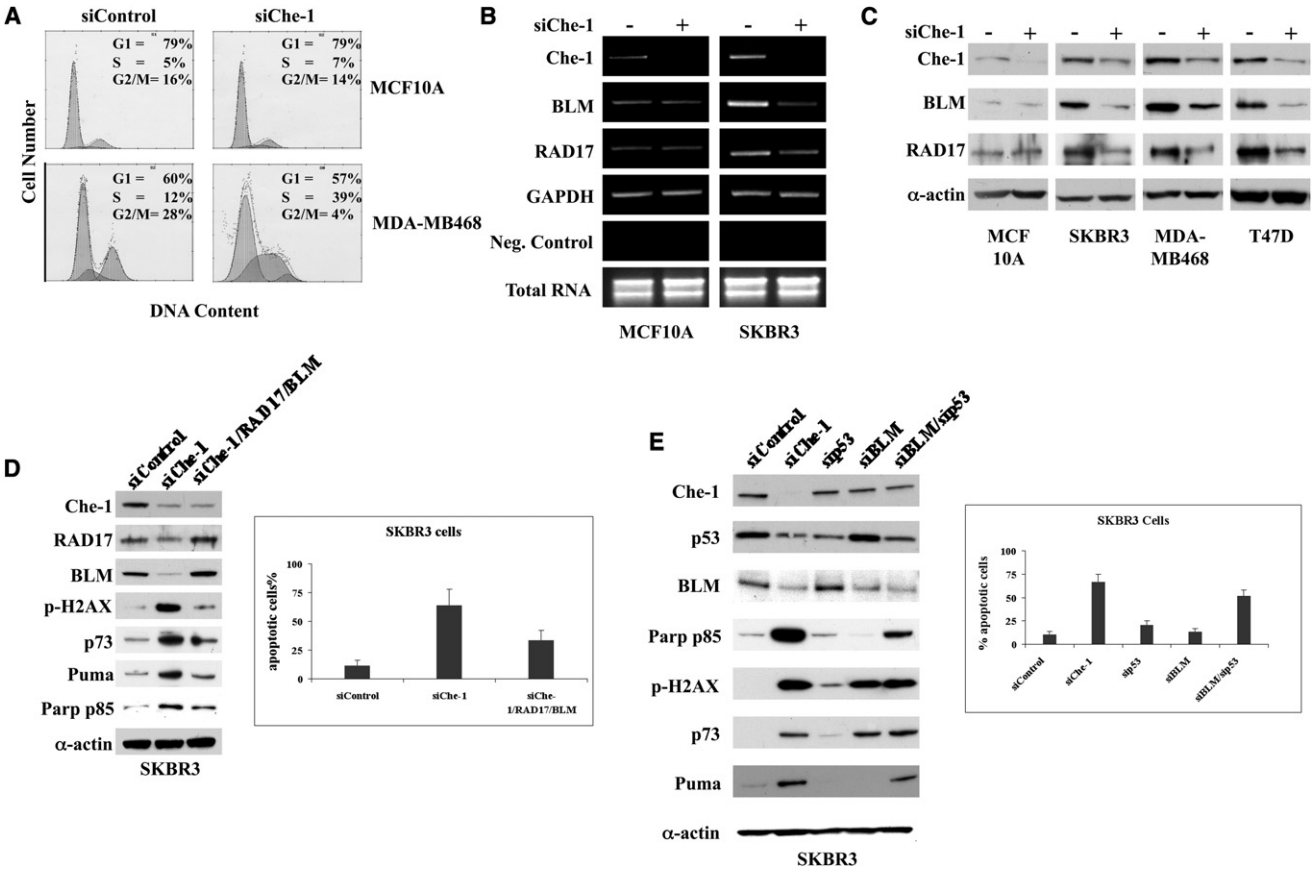

Figure 6. Che-1 Is Involved in DNA Repair

(A) MCF10A and MDA-MB468 cells were transiently transfected with siRNA GFP (siControl) or siRNA Che-1 (siChe-1). After 12 hr, cells were fixed and stained with propidium iodide (PI) and analyzed for DNA content.

(B) RT-PCR analysis of expression of the indicated genes from MCF10A and SKBR3 cells transiently transfected with siRNA GFP (–) or siRNA Che-1 (+).

(C) WB analysis with the indicated Abs of TCEs from the indicated cells transfected as in (B).

(D) SKBR3 cells were transiently transfected with siRNA GFP (siControl), siRNA Che-1 (siChe-1), or siChe-1 and BLM and RAD17 expression vectors. WB analysis of TCEs with the indicated Abs is shown on the left. Cell death analysis assayed by trypan blue staining is shown on the right. Percentages represent trypan blue incorporating cells. Data are presented as the mean ± SD from three independent experiments performed in duplicate.

(E) SKBR3 cells were transiently transfected with the indicated siRNA. WB analysis of TCEs with the indicated Abs is shown on the left. Cell death analysis assayed by trypan blue staining is shown on the right. Percentages represent trypan blue incorporating cells. Data are presented as the mean ± SD from three independent experiments performed in duplicate. See also Figure S4.

(0.2 cm^3), animals were subdivided into groups and either treated or untreated with Dox (2.0 g/l) for 5 weeks. At the end of treatments, all the animals were sacrificed and tumor excited. Although similar experiments showed that mtp53 depletion reduces tumor growth ability and chemoresistance (Bossi et al., 2008), results from this analysis showed that with respect to control animals (ind-si/Che-1 – Dox, ind-si/Control +/– Dox) ablation of Che-1 protein deeply impacts on tumor growth, producing a complete remission of formed tumor (Figures 7C and 7D). Histological examination of excised tumors revealed that induced Che-1 depletion displayed ample necrosis, scarred tissue and scanty mononuclear cell infiltrates respect to ind-si/Che-1 – Dox and ind-si/Control +/– Dox control tumors (Figure 7E). Hence, abrogation of Che-1 expression by RNAi completely inhibited MDA-MB468 tumor malignancy and strongly suggests Che-1 as a possible antineoplastic therapeutic target.

DISCUSSION

The gain of function of mtp53 promotes tumor formation and drug resistance, contributing to the poor prognosis of cancer patients. Therefore, several therapeutic rationales targeting mt53 activity are currently under investigation including attempts to inhibit mtp53 expression. In the present study, we provide evidence that Che-1 is required for mtp53 expression in human cancer cells, and that its depletion induces a specific apoptotic program in these cells.

We have previously reported that in response to DNA damage, Che-1 is stabilized and promotes wild-type *p53* gene transcription, contributing to the maintenance of the G_2/M checkpoint (Bruno et al., 2006). Here we show that Che-1 is required for mtp53 expression in several human cancer cell lines, and in such a way can contribute to mtp53 "gain of function." Furthermore, inhibition of Che-1 leads to DNA damage-dependent p73

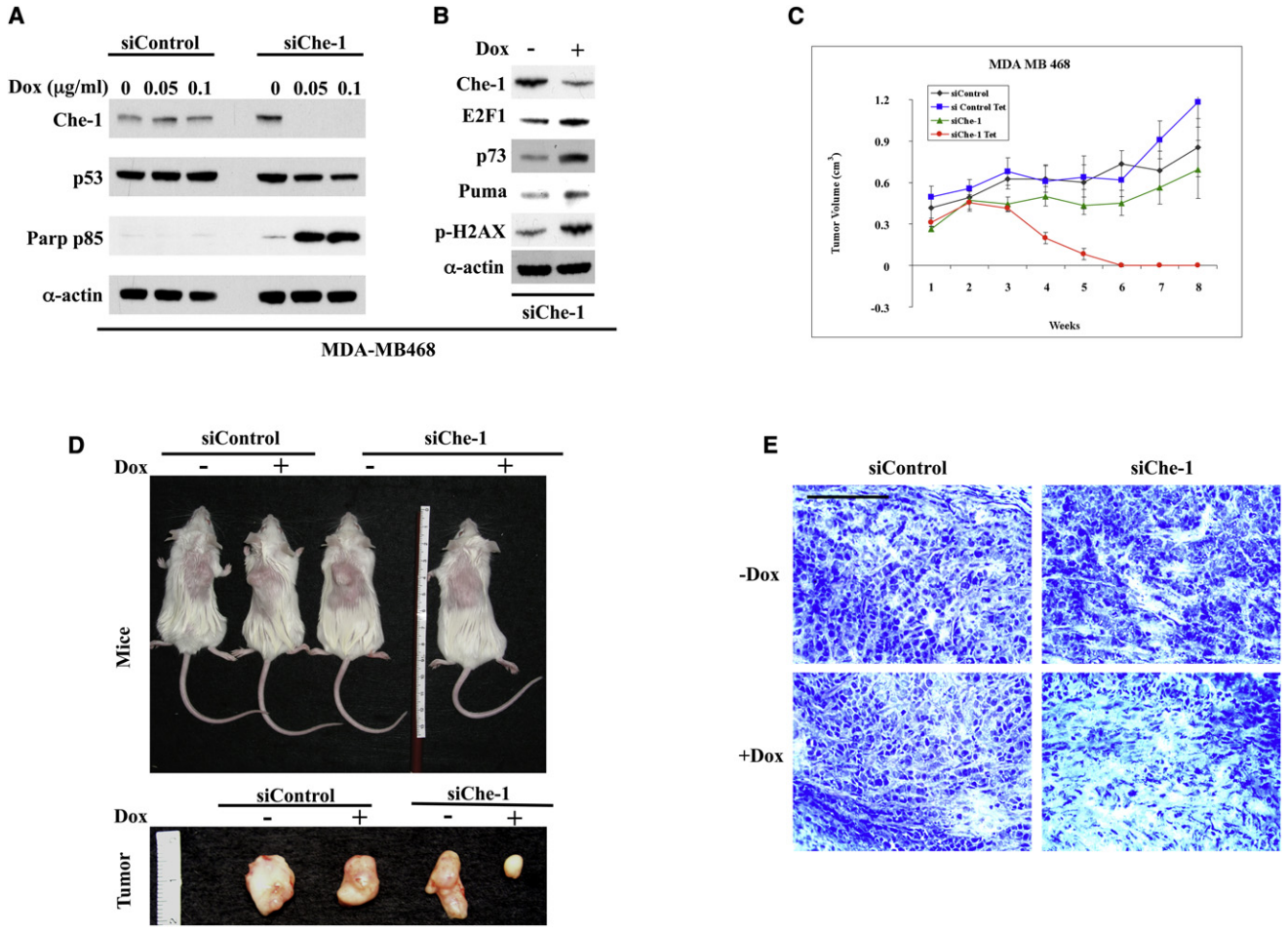

Figure 7. Che-1 Depletion Suppresses Tumor Growth in a Mouse Xenograft Model

(A) MDA-MB468 cells were infected with LV-THsh/Che-1 (siChe-1) or LV-THsh/Control (siControl) and LV-tTR-KRAB lentiviruses. For monitoring inducible shRNA expression, different concentrations of doxycycline (Dox) were added to the cell medium. Five days later, cells were harvested and processed for WB analysis with the indicated Abs.

(B) TCEs from MDA-MB468 cells induced (+Dox) or not (−Dox) were analyzed by WB with the indicated Abs.

(C) Engineered MDA-MB468 ind-siChe-1 and MDA-MB468 ind-siControl cell lines were implanted into subcutaneous of *Scid/Scid* mice, and animals treated as reported (see Experimental Procedures). Animals were monitored weekly as reported in Experimental Procedures. Data are presented as the mean ± SD from three independent experiments performed with different groups of ten mice.

(D) Representative mice (top) or excised tumors (bottom) from induced (+Dox) or not (−Dox) MDA-MB468 ind-siChe-1 and MDA-MB468 ind-siControl implanted cells.

(E) Sections of tumors from mice injected and treated as in (C) were stained with hematoxylin and eosin. The scale bar represents 100 μm.

activation and apoptosis. We also show that Che-1 is not only a component of DNA damage response, but is involved in DNA repair mechanisms, regulating the expression of important genes such as BLM and RAD17, suggesting that cancer cells carrying mtp53 require these proteins to proliferate. Finally, we provide evidence that the constitutive deregulation of Che-1 abolishes the tumorigenicity of cancer cells in an animal model.

Our results report that Che-1 is accumulated and recruited onto the *p53* promoter in cancer cells but not in primary breast fibroblasts (Figures 1A and 1B). These findings are in agreement with the notion that DNA damage response is, at least in part, chronically activated in human cancer cells (Bartkova et al., 2005; Gorgoulis et al., 2005). Moreover, several reports have described an induction of genetic instability by gain of function mtp53 (Murphy et al., 2000; Murphy and Rosen, 2000; Song et al., 2007), that might induce Che-1 stabilization and thereby activation of p53 expression.

We show that inhibition of Che-1 by siRNA strongly decreases mtp53 levels in several cancer cell lines. This effect is essentially due to transcriptional control of p53 by Che-1 (Figure 1C) but we cannot exclude the possibility that Che-1 might exert a translational control of p53 expression. Notably, ablating Che-1 expression triggers induction of Puma and apoptotic cell death. These events were not observed when mtp53 was depleted, leading to the hypothesis that apoptosis is activated by other mechanism/s. Nevertheless, although Che-1 exhibits an anti-apoptotic activity, its depletion did not trigger apoptosis in normal cells or in tumor cells carrying either wtp53 or lacking

p53 expression, thereby suggesting that this phenomenon requires mtp53 downregulation. Accordingly, Che-1 RNAi in MDA-MB231 cells did not induce either mtp53 inhibition or apoptosis, and mtp53 overexpression rescued apoptosis in Che-1-depleted SKBR3 and MDA-MB468 cells.

Our findings demonstrate that Che-1 inhibition induces p73 transactivation and its recruitment on the *Puma* promoter. This activity is required for induction of apoptosis. Relocalization of p73, Puma induction and PARP-1 cleavage all occur within 24 hr of siRNA transfection. This interval presumably reflects the time necessary for Che-1 and mtp53 degradation, for p73 transactivation and its release from mtp53/p73 complex, and finally for assembly of an active p73 complex at the *Puma* promoter. Therefore, it is reasonable to speculate that p73-mediated cell death after Che-1 depletion may represent the cumulative effects of increased p73 levels and concomitant decreased mutant p53- mediated inhibition.

It has been previously demonstrated that p73 is regulated in response to DNA damage through Chk1- and Chk2-mediated E2F1 activation and stabilization (Gonzalez et al., 2003; Urist et al., 2004; Pediconi et al., 2003). Accordingly, here we show that Che-1 depletion in tumor cells expressing mtp53 induces endogenous checkpoint activation, E2F1 stabilization, and its relocalization onto *p73* promoter. In addition, Che-1 overexpression was found to strongly inhibit E2F1-mediated p73 transactivation (not shown). Given that Che-1 binds the E2F1 complex in a proliferative pathway (Bruno et al., 2002), it might also be possible that Che-1 RNAi induces the release of E2F1 from cell cycle promoters, allowing its accumulation on the *p73* promoter.

Recent findings have indicated that mutant forms of p53 can inactivate critical DNA damage-response pathways, by removing the MRN complex from DNA double strand breaks (Song et al., 2007). Thus, it is possible that downregulating mtp53 expression, Che-1 depletion restores a functional checkpoint activation and apoptosis. Nevertheless, the observation that MDA-MB468 Che-1 depleted cells exhibit S phase accumulation and a reduced BrdU incorporation, suggests that DNA replication stress might be involved. Consistent with this hypothesis, Che-1 was found to regulate transcription of several genes involved in DNA repair and DNA damage response. Interestingly, among the Che-1-regulated genes, we identified BLM and RAD 17, whose role in response to DNA replication stress is well established (Wu, 2007; Wang et al., 2006). In agreement with genomic instability observed in tumor cell expressing mtp53, we found increased levels of BLM and RAD17 proteins in these cells (Figure 6C) and in $p53^{-/-}$ H1299 cells stably overexpressing mtp53 (Figure S4F). Che-1 inhibition decreased expression of these genes at both RNA and protein level, and additional experimental data will provide more evidence to better characterize the mechanisms by which Che-1 regulates the transcription of these genes. Of note, Bloom's syndrome cells exhibit endogenous checkpoint activation related to DNA replication abnormalities (Rassool et al., 2003; Li et al., 2004; Rao et al., 2007), and *BLM/RAD17* double knockout chicken DT 40 cells showed an increased rate of DNA lesions that cause spontaneous cell death (Nishino et al., 2008). Strikingly, restoration of BLM and RAD17 expression in Che-1 depleted cancer cells almost completely reverted DNA damage and apoptotic program induced by Che-1 depletion (Figure 6D and

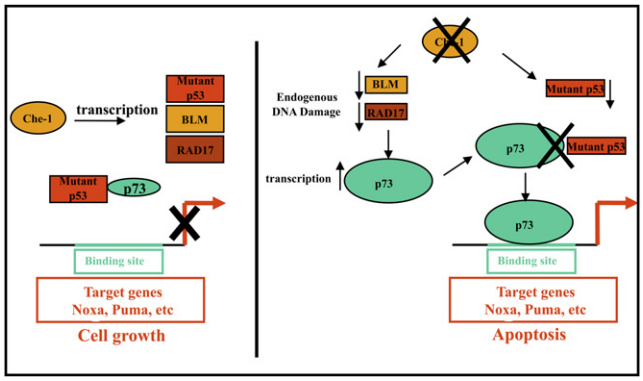

Figure 8. Model to Explain the Effects of Che-1 Depletion in Breast Cancer Cells Carrying Mutant p53

Che-1 inhibition decreases mutant p53 cellular levels weakening its gain of function. In addition, loss of Che-1 produces downregulation of important genes involved in replication checkpoint such as BLM and RAD17. This results in activation of DNA damage response, E2F1 stabilization, and transactivation of p73. Therefore, simultaneous modulation of p73 and mutant p53 levels allows the recruitment of p73 onto proapoptotic gene promoters and induces cell death.

Figure S4G), whereas simultaneous depletion of BLM and mtp53 induced DNA damage and apoptotic process (Figure 6E and Figure S4H). Together, our findings enable us to propose a model in which Che-1 is required for ensuring expression of both gain of function mtp53, and at the same time BLM and RAD17, for allowing cell proliferation, even in the presence of genomic instability (Figure 8). Therefore, although mtp53 knockdown reduces resistance to anticancer drugs (Bossi et al., 2006; Bossi et al., 2008), Che-1 depletion induces apoptosis without any genotoxic treatment (Figure 8). Consistent with this model, in vivo experiments performed by an inducible RNAi system demonstrate that conditional depletion of Che-1 deeply impacts on tumor growth, thereby identifying a therapeutic approach that, through allowing simultaneous modulation of p73 and mtp53 levels, might be used to target the large fraction of human tumors that harbor p53 mutations.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and Analysis

SKBR3, MDA-MB468, MDA-MB231, BT549, T47D, and ZR75-1 breast cancer cell lines were all cultured in RPM1 1640 with 10% fetal calf serum. H1299 human lung carcinoma cells and 293T packaging cells, kindly provided by Dr. S. Iacovelli were cultured in D-MEM high glucose with 10% FBS. MCF10A primary breast epithelial cells (a gift from Dr. Segatto) were cultured in Ham's F-12 medium with 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, and 20 ng/ml EGF. Cytofluorimetric analysis of cellular DNA content was performed on propidium iodide stained cells by an Epics XL Analyzer (Coulter Corporation). The following plasmids were used in transfection experiments: pcDNA3-BLM (kindly provided by Dr. I.D. Hickson), pcDNA3-RAD17 (a gift from Dr. P. Perego), pcDNA3-p53His175, and pcDNA3-p53His273 (Strano et al., 2000). Transfections were carried out by Lipofectamine Plus (Invitrogen) following the manufacturer's instructions. TUNEL assays were performed by using TUNEL Apoptosis Detection Kit (Millipore) following the manufacturer's instructions.

Cell Extracts and Western Blot

Cell extracts were prepared as previously described (Bruno et al., 2006). Solubilized proteins (25 μg) were resolved on Mops NuPAGE precast 4%–12% gels

(Invitrogen). Western blotting was performed using the following rabbit polyclonal antibodies: anti-Che-1 (Fanciulli et al., 2000), PARP-1 p85 fragment (Promega), p73 (Bethyl), Puma (Calbiochem), BLM (Cell Signaling), and CHK2 phospho thr68 (Cell Signaling). Mouse monoclonal antibodies anti-p53 (DO1), E2F1 KH95 (Santa Cruz), α-actin (Sigma), Rad17 (Santa Cruz), ATM phospho ser1981 (Rockland), and γH2AX phospho ser139 (Upstate) were also used. Secondary antibodies used were goat anti-mouse and goat anti-rabbit, conjugated to horseradish peroxidase (BioRad). Immunostained bands were detected by the chemiluminescent method (Amersham).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays were performed as previously described (Bruno et al., 2002) with the following rabbit polyclonal antibodies: anti-Che-1, E2F1 C20 (Santa Cruz), and p73 (Bethyl). In each experiment, signal linearity was ensured by amplifying increasing amounts of template DNA. Generally, DNA representing from 0.005% to 0.01% of the total chromatin sample (input) or from 1% to 10% of the immunoprecipitated was amplified using promoter-specific primers. Immunoprecipitations with no specific immunoglobulins (Santa Cruz) were performed as negative control.

RNA Isolation, QRT-PCR, and RT-PCR Analysis

Cells were harvested 36 hr after transfection and total RNA isolated using TRIZOL reagent (Invitrogen) in accordance with the manufacturer's instructions, and the first-strand cDNA was synthesized with the Thermo Script RT-PCR kit (Invitrogen) in accordance with the manufacturer's instructions. Applied Biosystems 7500 system SDS software was used for real-time PCR analysis. The analysis was performed with SYBR-green I fluorescence (Applied Biosystems). Quantification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (as an internal control for gene expression in the cells) was performed with TaqMan Human GAPDH Control Reagents (with VIC Probe, Applied Biosystems).

For semiquantitative RT-PCR analysis, RT-PCR was performed with a Platinum quantitative RT-PCR kit (Invitrogen) in accordance with the manufacturer's instructions. PCR products were separated onto 1.5% agarose gel.

siRNA

The 22-nucleotide siRNA duplexes corresponded to nucleotides 1062–1083 and 1473–1492 of human Che-1 sequence, to nucleotides 773–794 of human p53 sequence, and to nucleotides 122–143 of the negative control green fluorescent protein (GFP) sequence were in vitro synthesized by Silencer siRNA construction kit (Ambion) following manufacturer's instruction. siRNA p73 duplexes were purchased from QIAGEN. RNA interference was performed as previously described (Bruno et al., 2002). siRNA-mediated interference experiments of E2F1 and BLM expression were performed by transfecting SMART pool specific or nonspecific control pool double-stranded RNA oligonucleotides (Millipore) using Lipofectamine Plus (Invitrogen).

Design and Cloning of shRNA

The following oligos were annealed and cloned in pLV-THM vector (Wiznerowicz and Trono, 2003) MluI/ClaI (Boehringer Mannheim, Germany) digested, generating the new lentiviral vectors pLV-TH si/Che-1 and pLV-TH si/control.

Si/Che-1 oligo sequences (nucleotides 824–842): 5′-gatccccAAAGTTTCTGAGGAAGTGGttcaagagaCCACTTCCTCAGAAACTTTtttttggaaa-3′ and 5′- agctttccaaaaaAAAGTTTCTGAGGAAGTGGtctcttgaaCCACTTCCTCAGAAACTTTggg-3′.

Si/control oligo sequences: 5′-cgcgtCTATAACGGCGCTCGATATttcaagagaATATCGAGCG-CCGTTATAGtttttggaaat-3′ and 5′-cgatttccaaaaaCTATAACGGCGCTCGATATtctcttgaaATA-TCGAGCGCCGTTATAGa-3′.

Viral Vectors

Lentiviral vectors pLV-THM and pLV-tTR-KRAB (Wiznerowicz and Trono, 2003) were produced by transient transfection in 293T cells according to standard protocols (Zufferey et al., 1997). In brief, subconfluent 293T cells were cotransfected with 20 μg of a plasmid vector, 15 μg of pAX2, and 6 μg of pMD2G-VSVG by calcium phosphate precipitation (GIBCO-BRL). After 6–8 hr, medium was replaced with fresh medium (6.0 ml/plate) supplemented with 1.0 mM Sodium Pyruvate (GIBCO-BRL). Lentiviruses were harvested 48 hr later, centrifuged 5 min at 3,000 RPM, aliquoted and stored at –80°C. Lentiviral stocks were titered following standard protocols (Wiznerowicz and Trono, 2003), and routinely a viral titer of 10^6 transducing units per ml was achieved.

Cellular Transduction

For conditional RNA interference, MDA-MB468 cells were plated in 24-well plate (3.0×10^4 cells/well). Sixteen hours later, medium containing LV-THsh/Che-1 (indsi/Che-1) or LV-THsh/Control (indsi/control) and LV-tTR-KRAB lentiviruses supplemented with 8.0 μg/ml of polybrene was added to the cells. After 16 hr of incubation, cells were washed and replenished with fresh medium. Ninty-six hours later part of the cells were processed for western blot analysis. For monitoring shRNA expression upon doxycycline hydrochloride (Dox) (D9891, Sigma-Aldrich), the newly generated MDA-MB468-indsi/Che-1 cells were plated and 24 hr later different concentrations of Dox were added to the medium. Dox was added every 3 days. Five days later, cells were harvested and processed for western blot analysis.

In Vivo Experiments

Forty-day-old immunodeficient *Scid*/*Scid* female mice (Charles River Laboratories, Lecco, Italy) in groups of ten were maintained in a sterile environment. Engineered MDA-MB468 cells were injected (1.5×10^7 cells/mouse) with Matrigel (BD Biosciences) in the intrascapular area. Doxycycline was delivered to the mice through drinking water (tap water + 3.0% sucrose [Sigma]) in dark stained bottles, and renewed every 4 days. MDA-MB468 xenograft tumor growth delay was monitored twice a week by caliper measurements, tumors volumes (TV [cm^3]) were estimated by formula: $TV = a \times (b^2)/2$, where a and b are tumor length and width respectively in cm. At the end of treatments animal were sacrificed in accordance with standard protocols, tumors excised, and sections frozen in liquid nitrogen. Sections were then conserved at –80°C for further analyses. All the procedures involving animals and their care were approved by the Ethical Committee of the Regina Elena Cancer Institute, (Prot. CE/572/07), and were conformed to the relevant regulatory standards in accordance with the Italian legislation.

ACCESSION NUMBERS

All microarray raw data tables have been deposited at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE20622 (submitter M.F.).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, one table, and four figures and can be found with this article online at doi:10.1016/j.ccr.2010.05.027.

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