Tip60 and p400 are both required for UV-induced apoptosis but play antagonistic roles in cell cycle progression

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The histone acetyl transferase Tip60 (HTATIP) belongs to a multimolecular complex involved in the cellular response to DNA damage. Tip60 participates in cell cycle arrest following DNA damage by allowing p53 to activate p21CIP (p21) expression. We show here that Tip60 and the E1A-associated p400 protein (EP400), which belongs to the Tip60 complex, are also required for DNA damage-induced apoptosis. Tip60 favours the expression of some proapoptotic p53 target genes most likely through the stimulation of p53 DNA binding activity. In contrast, p400 represses p21 expression in unstressed cells, thereby allowing cell cycle progression and DNA damage-induced apoptosis. Tip60 and p400 have thus opposite effects on p21 expression in the absence of DNA damage. We further found that this antagonism relies on the inhibition of Tip60 function by p400, a property that is abolished following DNA damage. Therefore, taken together, our results indicate that Tip60 and p400 play distinct roles in DNA damage-induced apoptosis and underline the importance of the Tip60 complex and its regulation in the proper control of cell fate.

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Introduction

In higher eukaryotes, DNA damages such as double-strand breaks are recognised and repaired by specialised machineries. The signal given by the DNA damage leads to the activation of specific checkpoints (such as the G1/S or the G2/M checkpoints) resulting in the appropriate cell response (Sancar et al., 2004). Progression into the cell cycle is stopped until damages are repaired, or, if the damages are too important, cells can enter the process of apoptosis. Defects in this DNA damage response can result in the accumulation of mutations, which in turn can lead to cell transformation and cancer.

The ATM/ATR pathway plays a critical role in the G1/S and G2/M checkpoints induced in response to DNA damage. The G2/M checkpoint is activated through the phosphorylation of the cdc25 phosphatase resulting in its binding to 14-3-3 proteins and its subsequent nuclear export (Sancar et al., 2004). Consequently, cyclin-dependent kinases remain phosphorylated and inactive, and cells cannot proceed to mitosis. Activation of the G1/S checkpoint by the ATM/ATR pathway is largely dependent on the p53 tumour suppressor (Sancar et al., 2004). P53 is involved in both cell cycle arrest and apoptosis following DNA damage. P53 plays its role, at least in part, through the activation of specific genes. It binds to specific target DNA sequences, which are located in the promoters of genes involved in cell cycle arrest in G1, such as the gene encoding the cyclin/cdk inhibitor p21 (el-Deiry et al., 1993), or genes involved in apoptosis, such as genes encoding the Fas death receptor or the mitochondrial protein Bax (Miyashita et al., 1994; Muller et al., 1998). In the absence of DNA damage, p53 activity is repressed in part by the oncoprotein Mdm2, which binds directly to the p53 activation domain (Momand et al., 1992). Mdm2 is a ring-finger dependent E3 ubiquitin ligase, which ubiquitinates p53 and thereby targets it to proteosomal degradation (Honda et al., 1997; Kubbutat et al., 1997). In addition, Mdm2 suppresses the transcriptional activity of p53 (Momand et al., 1992). Moreover, recent results indicate that Mdm2 can be recruited to p53 binding sites to mediate transcriptional repression through histone ubiquitination (Minsky and Oren, 2004). Following DNA damage, activation of the ATM/ATR pathway results in p53 phosphorylation on multiple sites and in the inhibition of Mdm2 binding (Meek, 2004). P53 is then stabilised and its transcriptional activity is induced. What mediates the decision between a p21-dependent G1 arrest or apoptosis is still not completely clear for the moment (Meek, 2004).

The histone acetyl transferase (HAT) Tip60 also plays a major role in the DNA damage response. Indeed, we and others found that it is required for G1 arrest following actinomycin D treatment or DNA double-strand breaks and for apoptosis following double-strand breaks (Ikura et al., 2000; Berns et al., 2004; Legube et al., 2004). Tip60 has been cloned as a protein of 60 kDa, which binds to the HIV Tat protein (Kamine et al., 1996). Sequence analysis indicates that it belongs to the MYST (from MOZ-Ybf2/Sas3-Sas2-Tip60) family of HATs, which is conserved from yeast to human (Utley and Cote, 2003). The so-called Tip60 complex, purified using an exogenous tagged Tip60, contains various enzymatic activities, including two helicases of opposite polarity, Tip49a and Tip49b, and a chromatin remodelling enzyme from the SWI/SNF family of ATPases, the E1A-associated p400 protein (Ikura et al., 2000; Fuchs et al., 2001). That this
complex is relevant to Tip60 function is indicated by the finding that the complex is much more efficient in acetylating histones than purified Tip60 by itself (Ikura et al, 2000). Moreover, the Tip60 complex is structurally and functionally conserved from yeast to human (Ceol and Horvitz, 2004; Doyon et al, 2004). Because of the importance of histone modifications in transcription, many studies have investigated the role of the Tip60 and the Tip60 complex in transcriptional control. Tip60 has been found to function as a co-activator for many transcription factors (Brady et al, 1999; Hlubek et al, 2001; Baek et al, 2002), and binding of many members of the Tip60 complex (including Tip60 itself) to some E2F or c-myc regulated-promoters correlates with their transcriptional activation (Frank et al, 2003; Taubert et al, 2004).

In addition to its role in transcription, recent results indicate that Tip60 and the Tip60 complex play a direct role in the cell response to DNA damage, in particular following DNA double-strand breaks. First, in yeast, the NuA4 complex, which is the orthologue of the Tip60 complex, is recruited to sites of DNA breaks through binding to phosphorylated H2A (Downs et al, 2004). Second, in Drosophila, the Tip60 complex is important for the loss of phosphorylated H2Av (a Drosophila histone H2A variant homologous to H2AX) foci following double-strand break repair (Kusch et al, 2004). Drosophila Tip60 (dTip60) acetylates phosphorylated H2Av, thereby favouring its exchange with unphosphorylated H2Av mediated by the Drosophila homologue of p400, Domino. Thirdly, Tip60 directly acetylates ATM and may therefore participate in the signalling pathways controlling the cell response to DNA double-strand breaks (Sun et al, 2005). Finally, Tip60 is a major component of the p53 pathway: We previously found that it is coregulated with p53, since it is also a target of the E3 ubiquitin ligase activity of Mdm2 and since its expression increases following UV irradiation (Legube et al, 2002). Moreover, its expression is required for both the p53-dependent G1 arrest and the expression of p53 dependent genes (Berns et al, 2004; Doyon et al, 2004; Legube et al, 2004).

Here, we observed that the expression of both Tip60 and another component of the Tip60 complex, p400, are required for apoptosis induced by UV irradiation. However, whereas Tip60 favours the expression of p53-dependent proapoptotic genes in agreement with its described role as a p53 cofactor, p400 had an opposite effect and functioned as a repressor of p21 and fas transcription in unirradiated cells.

This surprising result led us to investigate the molecular mechanisms involved. We found that Tip60 was required for efficient binding of p53 to its target promoters. Finally, we demonstrate that the role of p400 in apoptosis is due to its ability to repress p21 expression and therefore to allow cell cycle progression. By manipulating p400 levels, we were able to change cell fate from apoptosis to cell cycle arrest, pointing out the importance of the Tip60 complex and its regulation in the choice between cell cycle arrest or apoptosis following DNA damages.

Results

Tip60 expression is required for UV-C-induced apoptosis
To test whether Tip60 is important for apoptosis controlled by the p53 pathway, we knocked-down Tip60 expression in p53-positive U2OS osteosarcoma cells using specific siRNAs that we previously characterised (Legube et al, 2004). We reproducibly achieved more than 90% of transfection efficiency using siRNAs (data not shown). We then irradiated cells with UV-C in order to induce apoptosis. Cells transfected with control siRNAs underwent massive apoptosis when irradiated, as indicated by extensive chromatin condensation, nucleus size reduction (Figure 1A) and caspase-mediated PARP cleavage (Figure 1B). Knockdown of p53 reduced apoptosis induction (data not shown), indicating that UV-induced apoptosis is, at least in part, p53-dependent. In cells in which Tip60 was knocked-down, virtually no apoptosis could be observed either by direct dapi staining (Figure 1A) or by the analysis of PARP cleavage (Figure 1B), even at a high UV-C dose (80 J/m²). Moreover, this result was obtained using two siRNAs targeting two different regions of the Tip60 mRNA (Tip1 and Tip2, Figure 1B), indicating that it is unlikely to be due to ‘off target’ effects of Tip60 siRNAs. These data thus indicate that Tip60 expression is required for apoptosis following UV-irradiation. Importantly, Tip60 is also required for apoptosis induced by other types of DNA damage, since Tip60 knockdown also inhibited apoptosis induced in U2OS cells by doxorubicin (a topo II inhibitor) and by cisplatin (Supplementary data 1).

p400 and Tip49b are also required for UV-induced apoptosis
In mammals, Tip60 belongs to a multimolecular complex, the so-called ‘Tip60 complex’, which is generally believed to be the active form of the enzyme (Ikura et al, 2000). To test whether Tip60 controls UV-induced apoptosis within the Tip60 complex, we investigated the role of other subunits of the Tip60 complex. We transfected U2OS cells with siRNA targeting the E1A-associated p400 ATPase. This siRNA induced the silencing of p400 expression at the mRNA level.
Figure 2 p400 and Tip49b are also required for UV-induced apoptosis. (A) U2OS cells were transfected with the indicated siRNAs using oligofectamine. After 48 h, they were harvested by scraping. Total RNAs were extracted and reverse transcribed. The amount of p400 cDNAs was measured by real-time PCR, divided by the amount of P0 cDNA and calculated relative to 100% for cells transfected with the control siRNAs. (B) Same as in (A), except that whole-cell extracts were prepared, immunoprecipitated with the anti-p400 antibody and blotted using anti-p400 or anti-Tip49b antibody. Also shown is a Western blot monitoring Tip49b levels in input material (p400 Western blots did not work properly on whole cell extracts). (C) U2OS cells were transfected with the indicated siRNAs using oligofectamine. After 48 h, the transfected cells were UV-irradiated (20 or 80 J/m²) or not. After 4 h, Lämmlmi sample buffer was added to the transfected cells to ensure extraction of total proteins. Cell extracts were then analysed for the presence of PARP by Western blot.

(Figure 2A) as well as at the protein level, as shown by the disappearance of a faint 400 kDa band recognised by the anti-p400 antibody and of coprecipitating Tip49b (lower panel) in p400 immunoprecipitates (Figure 2B). We then irradiated cells and assayed caspase-mediated cleavage of PARP. We found that knockdown of p400 resulted in a significant decrease of PARP cleavage even at high UV dose (80 J/m²) (Figure 2C), indicating that p400 expression is required for UV-induced apoptosis. We also found that knockdown of Tip49b had a similar effect (Supplementary data 2). Taken together, these data demonstrate that at least three subunits of the Tip60 complex, Tip60 itself and the ATPases Tip49b and p400 are required for DNA damage-induced apoptosis.

Tip60 and p400 play opposite roles in the expression of p53-dependent genes

UV-induced apoptosis is largely controlled through the p53 transcription factor, which induces the transcriptional activation of proapoptotic target genes, such as genes encoding Fas or Bax (Miyashita et al., 1994; Müller et al., 1998). As Tip60 has already been shown to be involved in transcriptional activation of p53-responsive genes, we tested the effect of the knockdown of Tip60 and p400 on the expression of proapoptotic p53-responsive genes. We irradiated cells with 10 J/m² of UV and harvested them 16 h later to achieve maximal induction of p53-dependent genes (data not shown). Under these conditions, apoptosis induction was still observed (data not shown). We analysed by reverse transcription followed by quantitative PCR (Q-PCR) the expression of some p53 target genes, including the gene encoding p21, which is already known to be dependent on Tip60 expression (Berns et al., 2004; Doyon et al., 2004; Legube et al., 2004). Induction upon UV irradiation was observed for four p53 target genes (p21, fas, hdm2 and bax) (Figure 3), whereas three p53 target gene (PTEN, PIG3 and Apaf1) were not activated (Supplementary data 3), the reasons for these differences being unclear. Knockdown of Tip60 resulted in a decreased expression of these genes, both in unirradiated cells and following irradiation (Figure 3A and Supplementary data 3), indicating that Tip60 is required for their expression. Surprisingly, knockdown of p400 had a completely different effect: whereas bax, PTEN, PIG3, Apaf1 were unaffected (data not shown), p21, fas and hdm2 were activated to some extent upon p400 knockdown in unirradiated cells (Figure 3B). This activation is unlikely to be due to ‘off-target’ effects of the siRNAs, since a similar activation of p21 expression following p400 knockdown was recently described in IMR 90 primary human diploid fibroblasts (Chan et al., 2005). Thus, in unstressed cells, p400 represses the expression of at least three p53 target genes. P400 knockdown did not significantly induce the expression of these genes following UV irradiation (Figure 3B) (or after doxorubicin or cisplatin treatment (Supplementary data 1)), suggesting that the repressive effects of p400 were abolished following DNA damage. Importantly, similar results were obtained in MCF7 cells upon UV irradiation, at least for p21 expression (Supplementary data 4). Taken together, these results indicate that although the expression of Tip60 and p400 are required for UV-induced apoptosis, they play different roles in the expression of some p53-dependent genes. In addition, they suggest that the effects of Tip60 on apoptosis could be dependent, at least in part, on its ability to favour the expression of some proapoptotic p53 target genes, such as fas or bax.

Tip60 knockdown does not affect p53 activation by UV

We thus investigated further the mechanism by which Tip60 favours the expression of some p53-dependent genes. We first tested whether Tip60 knockdown affects p53 accumulation or activation following UV irradiation. Indeed, Tip60 knockdown is known to result in a decrease in p53 expression in uninduced U2OS cells (Legube et al., 2004). Furthermore, Tip60 expression has recently been shown to be required for efficient ATM activation following DNA doublestrand breaks (Sun et al., 2005). Analysis of p53 expression in transfected cells indicates that, as already shown (Legube et al., 2004), knocking down Tip60 slightly decreased p53 levels in uninduced cells (Figure 4A). However, following UV irradiation conditions similar to Figure 3, p53 accumulates to nearly equivalent levels in cells transfected by the control or by the Tip60 siRNAs. Accurate quantification of this experiment indicated that the decrease in p53 levels was lower than 1.2-fold in cells transfected by the Tip60 siRNAs following UV irradiation, indicating that Tip60 knockdown did not significantly affect p53 accumulation under our experimental conditions. We also analysed p53 activation by performing Western
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**Figure 3** Tip60 and p400 have opposite effects on the expression of p53-dependent genes. (A) U2OS cells were transfected by the C1 siRNAs (black bars) or the Tip siRNAs (grey bars) by electroporation. After 48 h, they were UV-irradiated or not (10 J/m²), as indicated, and harvested 16 h later by scraping. Total RNAs were extracted and reverse transcribed. The amounts of p21, fas, bax and hdm2 cDNA were measured by Q-PCR, divided by the amount of ribosomal phosphoprotein PO cDNA and calculated relative to 1 for cells transfected by the control siRNAs and not irradiated. A representative experiment is shown. (B) U2OS cells were transfected by the C1 siRNAs (black bars) or the p400 siRNAs (grey bars) by electroporation and treated as in (A). The amounts of p21, fas and hdm2 cDNA were measured by real time PCR and analysed as in (A). A representative experiment is shown.

Blot against various phosphorylated forms of p53. We found that p53 was phosphorylated at S15, S37 and S392 to normal levels in UV-irradiated cells transfected by the Tip60 siRNAs (Figure 4B), indicating that Tip60 knockdown does not inhibit all the signal transduction pathways leading to p53 activation following UV-C irradiation. Since phosphorylation of p53 on serine 15 in response to UV-C irradiation is mediated through the ATR pathway (Tibbetts et al, 1999), our data thus suggest that Tip60 expression, although involved in ATM activation (Sun et al, 2005), is not required for ATR activation. Altogether, although we cannot rule out the possibility that Tip60 is required for a signalling pathway leading to p53 activation through a post-translational modification we have not tested, these results thus suggest that Tip60 plays a role in the p53 pathway downstream of p53 activation, in agreement with our previous finding that Tip60 is required for exogenous p53 to activate efficiently the p21 encoding gene in the absence of DNA damage signalling (Legube et al, 2004).

**Physical interaction between Tip60 and p53**

Since Tip60 is an HAT, which is involved in transcriptional activation, we investigated whether it could function as a co-activator for p53, which means that it would be recruited by p53 on promoters to mediate transcriptional activation. We first tested whether Tip60 physically interacts with p53.GST pull down experiments using bacterially produced GST-fusion proteins and transfected cell extracts indicated that p53 and Tip60 can interact, at least in vitro (data not shown). Since Tip60 is expressed at low levels in cells and since Tip60 and p53 migrate in the vicinity of the immunoglobulin heavy chain, co-immunoprecipitation of endogenous proteins was not conclusive (data not shown). Therefore, to test whether p53 and Tip60 interaction can be detected in living cells, we raised a cell line derived from U2OS cells and expressing exogenous HA-tagged Tip60. Reverse transcription followed by quantitative RT–PCR indicated that these cells expressed about 30-fold more Tip60 mRNA than the original U2OS cells. We then irradiated or not these cells. UV irradiation led to an increase in the expression of both endogenous p53 and HA-tagged Tip60 (Figure 5A and B), which was expected since both p53 and Tip60 are known to be upregulated in UV-treated cells (Legube et al, 2002; Meek, 2004). Strikingly,
immunoprecipitation of endogenous p53 from UV-irradiated cells led to the co-immunoprecipitation of HA-tagged Tip60, indicating that both proteins interact in irradiated cells (Figure 5A). Moreover, less Tip60 was co-immunoprecipitated with p53 in un-irradiated cells, indicating that irradiation of cells induced the accumulation of the p53/Tip60 complex. Similar results were obtained when the immunoprecipitation was performed in the opposite way (Figure 5B). Note, however, that since the expression of the two proteins increased following irradiation, we cannot conclude from these experiments that their ability to interact is induced by irradiation. Nevertheless, these data indicate that Tip60 interacts with p53, in agreement with the hypothesis that it functions as a co-activator for p53.

**Tip60 is required for p53 binding on p53 target promoters**

If Tip60 functions as a co-activator for p53, then it should not only interact with p53 but also be recruited to p53 target promoters through this interaction. However, our efforts to observe directly Tip60 recruitment to p53-dependent promoters by chromatin immunoprecipitation (ChIP) were unsuccessful, even in the HA-TIP60 overexpressing cell line in which we could readily detect the p53/Tip60 interaction (data not shown). Since transcriptional activation by p53 is known to be regulated at the level of p53 DNA binding, we also tested the effect of Tip60 knockdown on p53 binding to its target promoters by ChIP. We could readily detect p53 binding on the p21 promoter and on the fas promoter, since these sequences were highly enriched in the p53 ChIPs compared to control (Figure 6). No p53 binding was observed on a sequence derived from the β-actin gene, which is not targeted by p53. P53 occupancy was lower on the fas promoter than on the p21 promoter in uninduced cells (Figure 6), certainly reflecting a difference in p53 affinity for the two promoters. As expected, p53 binding to both promoters increased following UV irradiation. Tip60 knockdown resulted in the decrease of p53 presence on the two promoters, both in unirradiated cells and following irradiation (Figure 6). Since Tip60 knockdown did not significantly affect p53 expression following UV irradiation (Figure 4A), this result indicates that knockdown of Tip60 indeed compromises the ability of p53 to bind to its target sequences on the fas and p21 promoters, at least following UV irradiation. Therefore, all together, these experiments indicate that the role of Tip60 in apoptosis is mediated, at least in part, by its ability to favour p53 binding to its target promoters.

**p400 knockdown induces a p21-dependent cell cycle arrest**

Whereas the effect of Tip60 knockdown on some p53-dependent proapoptotic genes could explain its role in DNA damage-induced apoptosis, the molecular mechanism by which p400 knockdown blocked apoptosis is clearly different, since p400 was not required for the expression of the proapoptotic p53 target genes we tested (see Figure 3). We thus intended to investigate the molecular basis of p400 action. We found that p400 knockdown led to an increased expression of p21 mRNA (see above, Figure 3) and protein (Figure 7A). Since p21 functions as an inhibitor of cyclin/cdkks, we also investigated the phosphorylation status of a known important target of cyclin/cdkks, the retinoblastoma protein, which is a critical regulator of G1/S progression. We found that p400 knockdown resulted in the appearance of a faster migrating form of the retinoblastoma protein, thereby
reflected its dephosphorylation (Figure 7B). We finally analysed the cell cycle distribution of the transfected cells and we found that p400 knockdown in unstressed cells resulted in a G1 and a G2 cell cycle arrest (Figure 7C). Importantly, this cell cycle arrest is dependent on p21 activation, since it is abolished by the cotransfection of p21 siRNA (Supplementary data 5). Taken together, these data indicate that p400, through the inhibition of p21 expression, allows appropriate Rb phosphorylation and proper cell cycle progression. Similar results were obtained for Tip49b (Supplementary data 2).

**p400 knockdown blocks apoptosis through cell cycle arrest**

Since p21 and hypophosphorylated Rb are potent inhibitors of apoptosis (Harbour and Dean, 2000; Seoane et al., 2002), we reasoned that the effect of p400 knockdown on apoptosis (Figure 2) could be related to its ability to induce p21 expression and accumulation of hypophosphorylated Rb. To test this possibility, we prevented p21 accumulation following p400 knockdown by co-transfecting p21 siRNAs. We first checked knockdown efficiency by reverse transcription followed by Q-PCR. We found that p400 and Tip60 knockdown efficiencies were not affected by the presence of p21 siRNAs (Figure 8A). The analysis of p21 mRNA levels showed that, as already observed, p400 and Tip60 knockdown had opposite effects on p21 expression (upregulation and downregulation, respectively), whereas transfection of p21 siRNAs prevented p21 accumulation following p400 knockdown (Figure 8B). Importantly, similar results were observed when p21 levels were analysed by Western blot (Figure 8C). We then irradiated transfected cells and we analysed PARP cleavage, reflecting apoptosis (Figure 8D). We found that, as already observed, knockdown of either Tip60 or p400 resulted in inhibition of UV-induced PARP cleavage. Strikingly, inhibition of p21 expression restored PARP cleavage in cells transfected by p400 siRNAs, but not in cells transfected by Tip60 siRNAs, indicating that inhibition of apoptosis by p400 knockdown is largely dependent on the induction of p21 expression. Moreover, this result also demonstrates that Tip60 and p400 are involved in DNA damage-induced apoptosis through separate mechanisms.

**p400 role in cell cycle control is Tip60-dependent**

Our results from Figure 3 indicate that p400 and Tip60 play antagonistic roles in p21 gene expression. Since p400 and
Tip60 belong to the same complex, we reasoned that one of these proteins could regulate the other one. To test this possibility, we transfected U2OS cells with the two siRNAs together, and we analysed p21 gene expression. We found that both at the mRNA (Figure 9A) and the protein level (Figure 9B), p21 expression in cells transfected by the two siRNAs were down to the levels observed in cells transfected by the Tip60 siRNA alone: importantly, since no effect of p400 knockdown could be observed in Tip60 knockdown cells (although the efficiency of p400 knockdown was not affected by the Tip60 siRNAs (data not shown)), the effects of p400 are entirely Tip60-dependent. This led us to investigate whether the effects of p400 knockdown on cell cycle progression were also Tip60-dependent: we found by analysing Rb phosphorylation (Figure 9C) or cell cycle distribution (Figure 9D) that, whereas Tip60 knockdown had no major effect by itself, it was able to reverse completely the effects of p400 knockdown. Thus, the effects of p400 knockdown on p21 gene activation and cell cycle progression are Tip60-dependent, indicating that p400 functions as a negative regulator of Tip60 in unstimulated cells.

Discussion

**Tip60 and the p53 pathway**

In this manuscript, we analysed the role of two members of the so-called Tip60 complex in the cellular response to DNA damage. Our results, together with previous findings (Berns et al., 2004; Legube et al., 2004) indicate that Tip60 is required both for p53 dependent cell cycle arrest and apoptosis following DNA damage. Importantly, our data (Figure 3 and Supplementary data 3) as well as data obtained by others demonstrate that Tip60 is important for the expression of many p53 target genes (p21, fas, bax, hdm2, Apaf1, PTEN, PIG3) (Berns et al., 2004; Doyon et al., 2004; Legube et al., 2004), indicating that it mediates its effects as a cofactor of p53. How can Tip60 affect p53 function? One possibility could be that Tip60 expression is required for a signalling pathway leading to p53 activation. Although we cannot definitively rule out this possibility, we think it is unlikely, since (i) we found that Tip60 expression is not required for some UV-inducible phosphorylation events on p53 (Figure 4); (ii) we and others previously found that Tip60 knockdown specifically affects the p53-dependent G1 arrest but not the p53-independent G2/M arrest following DNA damage (Berns et al., 2004; Legube et al., 2004), indicating that the pathway leading to the G2/M arrest is intact in Tip60 knocked-down cells; (iii) Tip60 is required for activation of the p21 promoter by overexpressed p53 in the absence of stress signalling (Legube et al., 2004). Thus, Tip60 likely functions downstream of p53 activation. Another important and still unresolved issue is whether the effect of Tip60 on p53-dependent transcription is direct or not. We show that Tip60 and p53 interact in a UV-inducible manner and it has previously been found that TRRAP, a component of the Tip60 complex but present in other complexes as well, binds to p53 and mediates transcriptional activation by p53 (Barlev et al., 2001; Ard et al., 2002), in agreement with the possibility that TRRAP/Tip60 directly regulates p53 activity. Since Tip60 is an HAT, which has been shown to function as a co-activator for many transcription factors, a tempting hypothesis is that Tip60 is recruited to its target promoters by p53 where it would mediate transcriptional activation by p53 through histone acetylation. Our results anyway indicate that Tip60 knockdown resulted in a decreased binding of p53 to its target sequences (Figure 6). The effect of Tip60 on p53-dependent genes expression can be explained by this finding, since transcription of these genes is dependent on p53 binding to their promoters.

What is the mechanism by which Tip60 allows p53 binding to the promoters? Interestingly, the DNA binding activity of p53 is known to be regulated by acetylation (Gu and Roeder, 1997; Luo et al., 2004). It is thus tempting to speculate that Tip60 directly acetylates p53, thereby inducing its ability to bind DNA. It is important to note that, in our hands, bacterially produced GST-Tip60 or Tip60 immunoprecipitated from cells were not able to acetylate recombinant bacterially expressed p53 in vitro (G Legube and D Trouche, unpublished).
results). The possibility still remains that specific post-translational modifications of p53 or Tip60 or the presence of additional co-factors are required for this acetylation to occur. Alternatively, Tip60 can affect the function of other HATs, such as CBP/p300, which are known to acetylate p53 (Gu and Roeder, 1997). Consistent with this possibility, we recently showed that Tip60 and p300 physically interact and that p300 regulates Tip60 expression (Col et al., 2005). Whether p300 properties are also modified through Tip60 binding remains to be investigated.

p400: a negative regulator of Tip60
Our data also show that p400 knockdown leads to cell cycle arrest, indicating that p400 expression is required for cell cycle progression. The role of p400 in cell cycle progression is due to its ability to repress the expression of p21, a major regulator of cyclin/cdk activity. What is the molecular mechanism involved? We found that p400 on the one hand and Tip60 and p53 on the other have antagonistic roles on p21, fas and hdm2 mRNA expression, since Tip60 or p53 knockdown decrease their expression whereas p400 knockdown stimulates it. Moreover, we observed that Tip60 knockdown results in lower binding of p53 to fas and p21 promoters. Strikingly, p400 knockdown has been shown to favour p53 binding to the p21 promoter (Chan et al., 2005), a finding that we confirmed in U2OS cells (Supplementary data 6). Thus, p400 and Tip60 have also antagonistic roles on p53 binding to the p21 or the fas promoter.

Importantly, we found that the effects of p400 knockdown on p21 and fas expression are entirely Tip60-dependent, indicating that p400 is a negative regulator of Tip60. Altogether, our data indicate that Tip60 favours p53 binding to the p21 or the fas promoter by an unknown mechanism (see above), and that the major role of p400 is to repress this function of Tip60. Consistent with such a mechanism, the effects of p400 are p53-dependent, since the phenotype induced by the p400 siRNA can be reversed, at least partially, by co-transfection of p53 siRNA (Supplementary data 7).

Since Tip60 and p400 are both present in the Tip60 complex, p400 could repress Tip60 function in the context of the Tip60 complex. Strikingly, the Tip60 complex is separated in two different complexes in yeast, the NuA4 complex containing the HAT Esa1, the orthologue of Tip60, and the SWR1 complex, containing the orthologue of p400 (Doyon et al., 2004). It is thus tempting to speculate that the orthologue of the SWR1 complex (i.e. a subcomplex including p400 and Tip49b) could function as a regulatory module within the Tip60 complex, controlling the function of a subcomplex orthologue to the NuA4 complex. Note, however, that we cannot rule out for the moment the possibility that the effect of p400 on Tip60 function is indirect.

Whatever the mechanism, we did not observe any stimulating effects of p400 knockdown on p21, hdm2 or fas expression in UV-irradiated (Figure 3) or doxorubicine- or cisplatinetreated cells (Supplementary data 1), although their expression is still dependent on Tip60. This result suggests that the repressive effects of p400 on Tip60 function are abolished upon DNA damage and that p400 is directly or indirectly a target of DNA damage-induced signal transduction pathways. Further works are required to clarify this point.

Role of the Tip60 complex in apoptosis
Our data together with published data demonstrate that Tip60 expression is required for p53-dependent cell cycle arrest and apoptosis following DNA damage. Here, we observed that two other members of the Tip60 complex, p400 and Tip49b, are also required for apoptosis. Whereas Tip60 controls apoptosis, at least in part, as a co-factor for p53, the primary role of p400 in apoptosis is mediated through the repression of p21 expression in unstressed cells. Indeed, p21 levels are a major determinant in the cell decision to stop proliferating or to enter apoptosis following DNA damage (Seoane et al., 2002). In our study, we were able to change the fate of irradiated cells from apoptosis to cell cycle arrest by manipulating p400 levels. It would be thus interesting to find signals that would lead to such an effect. Indeed, according to our findings, one could predict that cells with lower p400 expression would be less sensitive to DNA-damage induced apoptosis.

Our findings anyway indicate that members of the Tip60 complex play distinct roles in the control of DNA-damage-induced apoptosis, suggesting the existence of subcomplexes with different functions. In agreement with this possibility, a complex similar to the Tip60 complex but devoid of Tip60 has been described (Fuchs et al., 2001), suggesting that the presence of Tip60 within its complex is a regulated event. The relative levels of these subcomplexes are likely to play major roles in the control of cell fate following DNA damage. Clearly, a major challenge in the future is to characterise potential DNA damage-induced modifications of Tip60 subcomplexes formation, and the molecular mechanisms involved. Finally, the finding that members of the Tip60 complex are required for apoptosis following DNA damage indicates that loss of their activity can lead to the accumulation of heritable mutations. The possibility of pathological modifications of expression levels or activity of these proteins in cancer thus deserves investigation.

Materials and methods
Antibodies, siRNAs and plasmids
The anti-PARP, anti-S37P p53 and anti-S392P p53 antibodies were purchased from Cell Signaling Technology, the anti-p53 antibody (DO-1) from Santa Cruz, the anti-S15P p53 from Calbiochem, the anti-HDAC3 antibody (which recognises also HDAC1 and HDAC2) from Transduction Laboratories, the anti-HA antibody from Covance, the anti-Rb (G3-245) and anti-p21 antibodies from Pharmingen (Becton-Dickinson) and the anti-p400 antibody from Abcam. All secondary antibodies were purchased from Amersham. All siRNAs were purchased from Eurogentec and are described in Supplementary data 8. C1 does not recognise any human mRNA. Vectors for standard curves used in Q-PCR were constructed by inserting the various amplification products from cDNA preparations or ChIP inputs into pGEMT-easy vector (Promega), according to the manufacturer’s instructions. All PCR inserts were entirely sequenced.

Cell culture, transfections and UV-irradiation
U2OS cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with antibiotics and 10% fetal calf serum. A cell line overexpressing HA-Tip60 was constructed following transfection of pcDNA3 HA-Tip60 in U2OS and a 3 weeks selection in G418 (400 μg/ml). Ten resistant clones were picked and one of them was used for the experiments following analysis of recombiant Tip60 expression by Western blot and RT-Q-PCR analysis. For oligofectamine transfection, 150 000 cells were transfected with 4 μl of oligofectamine (Invitrogen), and 10 μl of the double-stranded
siRNAs (20 μM) according to the manufacturer’s instructions. For electroporation, cells were transfected in 200 μl of serum-free medium with 20 μl of siRNAs (20 or 100 μM) on a Biorad electroporation device set to 42 V/cm in a 20 μL-reaction containing 0.5 μg of oligo-dT or Random Primers (Promega), 1 x AMV RT buffer, 10 U of AMV Reverse Transcriptase (Promega), 40 U of rRNasin (Promega), 10 mM DTT and 0.5 mM deoxynucleotides (Promega). The reaction was stopped by incubating the samples for 15 min at 70 °C, and cDNAs were analysed by Q-PCR.

RNA extraction and reverse transcription
Total RNA was extracted using a RNeasy mini kit (QIAGEN) and eluted with 30 μl of RNase-free water. Two to five microlitres of each purified RNA preparation was reversed-transcribed for 30 min at 42 °C in a 20 μL-reaction containing 0.5 μg of oligo-dT or Random Primers (Promega), 1 x AMV RT buffer, 10 U of AMV Reverse Transcriptase (Promega), 40 U of rRNasin (Promega), 10 mM DTT and 0.5 mM deoxynucleotides (Promega). The reaction was stopped by incubating the samples for 15 min at 70 °C, and cDNAs were analysed by Q-PCR.

Chromatin immunoprecipitation assay
Formaldehyde was added to the culture medium to a final concentration of 1% and crosslinking was allowed to proceed for 10 min at room temperature. In order to stop the reaction, glycine was added to a final concentration of 0.125 M. After 5 min, cells were washed twice with cold PBS and harvested by scraping. Pelleted cells were first subjected to lysis in the following buffer: Pipes 5 mM pH 8, KCl 85 mM, NP-40 0.5%. This lysis was followed by homogenisation with a Dounce homogeniser and nuclei were harvested by centrifugation. Nuclei were then incubated in nuclear lysate buffer: 50 mM Tris pH 8.1, 10 mM EDTA, 1% SDS and sonicated five times for 10 s at a power setting of 5 and 50% duty cycle (Branson Sonifier 250), so as to obtain DNA fragments of about 500–1000 bp. Samples were diluted 10 times in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8.1, 167 mM NaCl) and subjected to a 45 min pre-clearing with 140 μl of previously blocked protein-A and protein-G beads (Sigma). Block- ing was achieved by incubating the agarose beads with 500 μg of BSA and 200 μg of herring sperm DNA for 3 h at 4 °C. Pre-cleared samples were incubated overnight at 4 °C with antibodies specific for p53 (10 μl) or without antibody as negative control. Immune complexes were then recovered by incubating the samples with 140 μl of blocked protein A/protein G beads for 2 h at 4 °C on a rotating wheel. Beads were washed once in dialysis buffer (2 mM EDTA, 50 mM Tris pH 8, 0.2% Sarkosyl) and four times in wash buffer (100 mM Tris pH 8.8, 500 mM LiCl, 1% NP-40, 1% NaDoc). Elution from the beads was achieved by incubation in elution buffer (1% SDS, 100 mM NaHCO₃) for 15 min. Crosslink was reversed by adding NaCl and RNase A to the samples and incubating overnight at 62 °C. After a 2 h-proteinase K treatment, DNA was purified with the GFX PCR kit (Amersham), and analysed by Q-PCR.

Quantitative PCR analysis
Q-PCR analysis was performed on a i-Cycler device (Biorad) using the plasmidium SYBR Green qPCR SuperMix (Invitrogen), according to the manufacturer’s instructions. All experiments included a standard curve. All samples were analysed in duplicates, and the mean and standard deviation were calculated. Primers are included in Supplementary data 8.

Analysis of cell cycle distribution
Cells were treated 30 min with BrdU (20 μM). Cells were then trypsinised, fixed with ethanol, and incubated for 30 min in 4 N HCl, 0.5% Triton. Cells were then extensively washed in PBS supplemented with 1% bovine serum albumin, incubated for 1 h with anti-BrdU antibody (BD Biosciences) and then 30 min with an FITC-conjugated secondary antibody (Sigma). Cells were then stained with propidium iodide (BD Pharmingen) and analysed by flow cytometry (FACSCalibur) on FL1 (anti-BrdU) and FL3 (propidium iodide) to determine their cell cycle distribution.

Supplementary data
Supplementary data are available at The EMBO Journal Online.

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