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Zbtb4 represses transcription of *P21CIP1* and controls the cellular response to p53 activation

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In response to stimuli that activate p53, cells can undergo either apoptosis or cell cycle arrest, depending on the precise pattern of p53 target genes that is activated. We show here that Zbtb4, a transcriptional repressor protein, associates with the Sin3/histone deacetylase co-repressor and represses expression of *P21CIP1* as part of a heterodimeric complex with Miz1. *In vivo*, expression of *ZBTB4* is downregulated in advanced stages of multiple human tumours. In cell culture, depletion of *ZBTB4* promotes cell cycle arrest in response to activation of p53 and suppresses apoptosis through regulation of *P21CIP1*, thereby promoting long-term cell survival. Our data suggest that Zbtb4 is a critical determinant of the cellular response to p53 activation and reinforce the notion that p21Cip1 can provide an essential survival signal in cells with activated p53.

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Introduction

The p53 tumour suppressor protein orchestrates a broad transcriptional programme in response to both physiological and environmental stress. Activation of p53 can result in either cell cycle arrest or apoptosis and this 'decision' is largely controlled by the exact pattern of p53 target genes that is regulated in response to a specific stimulus.

p53-induced cell cycle arrest is mediated by upregulation of *P21CIP1* which encodes an inhibitor of the Cdk2 kinase, and by suppression of cell cycle regulatory genes that include *CDC25C*, which encodes a phosphatase required for activation of Cdk1 (Vousden, 2006). p53-induced apoptosis is mediated by upregulation of a group of pro-apoptotic genes, most notably *NOXA* and *PUMA*. In addition, non-transcriptional functions of p53 at the mitochondrion have an essential pro-apoptotic function (Mihara *et al*, 2003). Importantly, induction of *P21CIP1* expression by p53 suppresses apoptosis in addition to inhibiting cell cycle progression (see Discussion). As a result, levels of p21Cip1 can be critical determinant of the outcome of p53 activation.

Several factors influence the choice of target genes that are induced in response to activation of p53 under specific circumstances (Vousden, 2006): the p53 protein itself integrates multiple signals that can favour either apoptosis or cell cycle arrest. For example, phosphorylation of serine residues 18 and 23 is required for induction of apoptosis in mice; similarly, phosphorylation of human p53 at residue 46 contributes specifically to activation of pro-apoptotic genes (Chao *et al*, 2006). Also, the ASPP proteins that bind to the p53 protein shift the transcriptional response towards the induction of pro-apoptotic genes and away from cell cycle arrest (Samuels-Lev *et al*, 2001). A second mechanism that allows for the specific regulation of individual p53 target genes is the combinatorial control of many targets of p53 together with other transcription factors. For example, induction of expression of the death receptor DR5 requires cooperation of p53 with NF-κB; also, the Slug repressor protein suppresses p53-dependent induction of *PUMA* in haematopoietic cells (Wu *et al*, 2005).

Conversely, induction of *P21CIP1* expression requires binding of Miz1 to the core *P21CIP1* promoter and, as a consequence, factors that control Miz1 can affect *P21CIP1* expression (Herold *et al*, 2002). One example of this is the Myc protein, which, when deregulated, represses p53-mediated induction of *P21CIP1* through binding to Miz1 and thereby favours apoptosis in response to p53 activation (Seoane *et al*, 2002). Re-expression of *P21CIP1* in Myc-transformed cells inhibits apoptosis and a point mutant of Myc that is unable to bind to Miz1 and therefore to repress *P21CIP1* is unable to induce apoptosis in human fibroblasts. This suggests that repression of *P21CIP1* can be essential for Myc-dependent apoptosis (Seoane *et al*, 2002).

At its amino-terminus, Miz1 contains a POZ protein–protein interaction domain, which can mediate both homo- and heterodimerization among POZ domain proteins. Miz1 heterodimerizes with Bcl-6 to suppress expression of *P21CIP1* during the class switching of immunoglobulin genes in B cells, when p53 is activated in response to the recombination events (Phan *et al*, 2005). We describe now a second POZ domain partner protein of Miz1, Zbtb4. Expression of *ZBTB4* is downregulated in advanced stages of human neuroblastoma and of multiple human solid tumours. Zbtb4 is a transcriptional repressor protein that heterodimerizes with Miz1, represses *P21CIP1* expression and inhibits cell cycle arrest in

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response to p53 activation. Conversely, loss of Zbtb4 inhibits apoptosis and favours cell cycle arrest and long-term survival in response to activation of p53. Our data suggest that the expression level of *ZBTB4* is an important determinant of the cellular response to p53 activation.

Results

Zbtb4 expression decreases in human tumours

In human neuroblastoma, advanced tumour stage and amplification of the *MYCN* gene are highly predictive for poor outcome. We have previously performed a microarray analysis of primary human neuroblastomas and identified expression signatures that are specific for individual tumour stages and for *MYCN*-amplified tumours (Berwanger *et al*, 2002). One of the genes that is downregulated in neuroblastomas with poor prognosis encodes Zbtb4, a POZ domain transcriptional repressor protein (Filion *et al*, 2006) (Figure 1A). To extend the microarray data, we measured expression of *ZBTB4* mRNA in 98 independent RNA samples of primary neuroblastomas and confirmed that *ZBTB4* is expressed in a stage-dependent manner, with expression being lowest in stage IV neuroblastoma ($P<0.01$) (Figure 1B). Expression levels of *ZBTB4* are inversely correlated with survival as seen in the Kaplan–Meier analysis (Figure 1C). As *ZBTB4* is expressed in a stage-dependent manner, expression levels of *ZBTB4* did not constitute an independent prognostic parameter in a multivariate analysis, which takes tumour stage into account (not shown).

Data obtained from a publicly available database of tumour expression profiles (www.oncomine.org) showed that downregulation of *ZBTB4* relative to normal tissue occurs in multiple human tumour entities, notably in breast, prostate and lung carcinoma and in glioblastoma (Figure 1D and Supplementary Table 1). In addition, *ZBTB4* is expressed in a stage-specific manner in glioblastoma and in breast, ovarian and prostate carcinoma, with expression being lowest in advanced tumour stages, similar to neuroblastoma (Figure 1E and Supplementary Table 2).

Biological effects of Zbtb4 downregulation

To understand how repression of Zbtb4 affects cell physiology, we used three distinct retroviral expression vectors that express a short hairpin RNA (shRNA) targeting *ZBTB4* (sh*ZBTB4-1,2,3*) or soluble siRNA oligonucleotides that target *ZBTB4* together with scrambled controls. RQ–PCR analysis showed that individual clones of SH-EP neuroblastoma cells stably infected with any of the three *shZBTB4* vectors had 2- to 3-fold lower levels of *ZBTB4* mRNA (Supplementary Figure 1A). Similarly, transfection of either SH-EP or SH-SY5Y

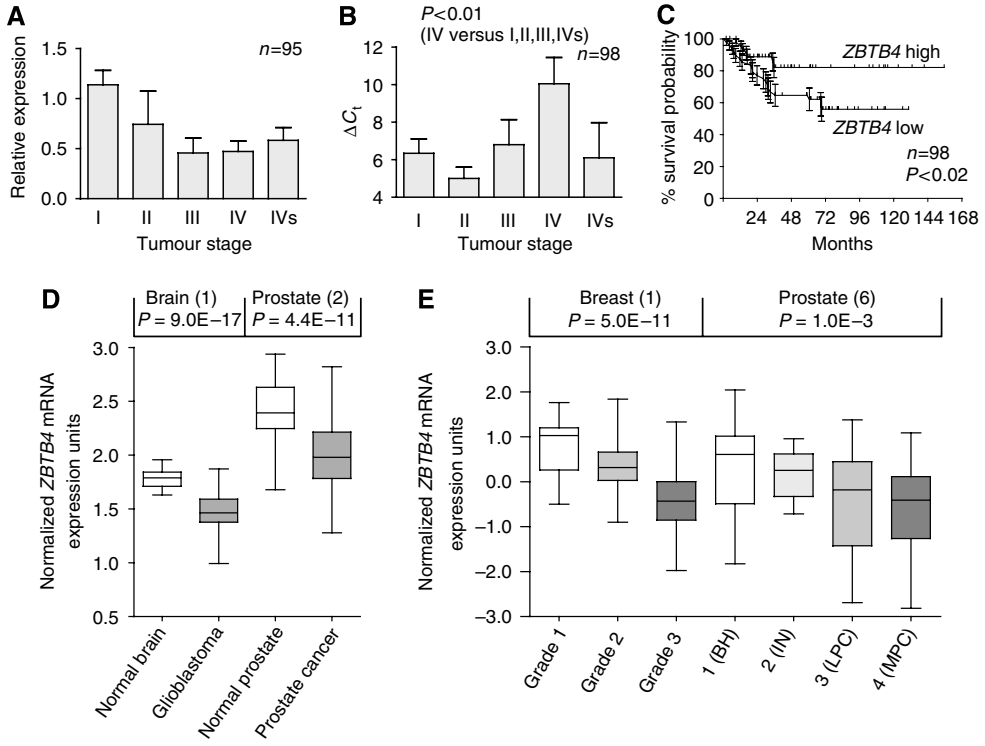


Figure 1 Expression of *ZBTB4* mRNA in human tumours. (**A**) *ZBTB4* is expressed in a stage-dependent manner in human neuroblastoma. The graph is a summary of microarray data of *ZBTB4* mRNA levels observed in different neuroblastoma tumour stages. The data are taken from the original analysis (Berwanger *et al*, 2002). Error bars represent standard error of the mean. (**B**) Confirmation of microarray data. The graph summarizes the RQ–PCR analysis of *ZBTB4* expression in an independent set of 98 primary neuroblastoma tumours. Error bars represent standard error of the mean. (**C**) Expression of *ZBTB4* correlates with patient survival. The graph documents the Kaplan–Meier analysis of neuroblastoma patients with *ZBTB4* mRNA expression levels above ('high') and below ('low') the mean expression level. (**D**) *ZBTB4* is repressed in glioblastoma and in prostate carcinoma relative to normal tissue. The data are taken from the studies summarized in Table 1, and were obtained from www.oncomine.org. The numbers refer to Table 1. In this and the following panel, data are presented as Box and Whisker graph with error bars representing the 5th and 95th percentile. (**E**) Stage-specific expression of *ZBTB4* in breast and prostate carcinoma. The data are taken from the studies summarized in Table 2. The plots are taken from www.oncomine.org (BH, benign hyperplasia; IN, intraepithelial hyperplasia; LPC, localized prostate carcinoma; MPC, prostate carcinoma metastasis).

neuroblastoma cells with soluble siRNAs targeting *ZBTB4* (si*ZBTB4*) reduced expression levels of *ZBTB4* approximately five-fold relative to control cells (Supplementary Figure 1B). Furthermore, expression of either of the sh*ZBTB4* vectors or transfection of si*ZBTB4* reduced expression levels of a HA-tagged Zbtb4 allele that was stably expressed in SH-EP cells (Supplementary Figure 1C).

Pools of SH-EP cells, which express sh*ZBTB4*, showed no significant change in the rate of proliferation relative to control cells under standard tissue culture conditions (Supplementary Figure 1D). Conversely, ectopic expression of Zbtb4 had little effect on cell growth at moderate expression levels and both stable cell clones and pools expressing moderately elevated levels of Zbtb4 could be established (data not shown and Figure 4). In contrast, expression of approximately 10-fold higher levels of Zbtb4 suppressed colony formation in several tumour cell lines and induced apoptosis, as revealed by the accumulation of cells with a subG1 ($<2n$) content of DNA (Supplementary Figure 1E).

To understand if downregulation of *ZBTB4* confers a selective advantage to tumour cells, we exposed SH-EP cells to a number of drugs that are used in the therapy of neuroblastoma patients, including cisplatin, etoposide, vincristine and retinoic acid (Estlin and Veal, 2003). All drugs suppressed colony formation of control SH-EP cells at the concentrations used in these experiments (Figure 2A). No difference was observed in the response of cells that stably express sh*ZBTB4* towards cisplatin, etoposide or retinoic acid (Figure 2A and data not shown). In contrast, cells expressing sh*ZBTB4* showed an enhanced long-term survival in response to vincristine (Figure 2A and Supplementary Figure 2A). This phenotype was observed in several cell clones with each of the three different shRNAs targeting *ZBTB4*, demonstrating that it did not reflect an off-target effect of an individual shRNA or an artefact due to the selection of an individual clone (Supplementary Figure 2A).

To demonstrate directly that depletion of *ZBTB4* provides a selective advantage in the presence of vincristine, we expressed sh*ZBTB4* together with a GFP marker in SH-EP cells (Figure 2B). The relative abundance of cells expressing either one of three sh*ZBTB4* vectors increased over time in the presence of vincristine relative to cells expressing a control shRNA or to cells expressing GFP alone. In contrast, Zbtb4-depleted cells had no survival advantage in the presence of DMSO, demonstrating that depletion of Zbtb4 provides a specific survival advantage in the presence of vincristine.

To understand the underlying mechanism, we performed FACScan experiments of SH-EP cells before and after addition of vincristine (Figure 2C and Supplementary Figure 2B). Vincristine induced apoptosis in control cells, as documented by the appearance of a cell population with a sub-G1 DNA content. In contrast, vincristine did not induce apoptosis in cells expressing any of the three sh*ZBTB4* vectors. Instead, over 80% of Zbtb4-depleted cells accumulated in the G1 phase of the cell cycle, demonstrating that vincristine induced a temporary arrest or delay in proliferation. To confirm these observations, we monitored cleavage of poly-ADP-ribose-polymerase (PARP), an early marker of apoptosis; consistent with the FACScan data, depletion of Zbtb4 reduced the extent of PARP cleavage in response to vincristine (Figure 2C). We concluded that depletion of Zbtb4 shifted the cellular response to vincristine from apoptosis to a temporary G1 arrest.

In contrast to etoposide or cisplatin, vincristine does not induce DNA damage but exerts its cytostatic effects through interaction with β-tubulin and interference with its function (Jordan *et al*, 1998) (see also Figure 4). At the low concentrations (5 nM) used in these experiments, vincristine does not disrupt the cytoskeleton but activates the tumour suppressor protein p53, potentially by facilitating its nuclear import, which depends on the microtubule-associated motor protein dynein (Giannakakou *et al*, 2000). Consistently, vincristine led to an accumulation of p53 and a p53-dependent induction of p21Cip1 protein expression as well as of *P21CIP1*, *NOXA* and *PUMA* mRNAs (Figure 3A and 5A). At higher concentrations (50 nM), vincristine disrupts the microtubular network, leading to a failure to execute mitosis (Jordan *et al*, 1998). Exposure of either control or Zbtb4-depleted cells to these elevated concentrations of vincristine suppressed colony formation (not shown) and led to an accumulation of cells with $4n$ DNA content, consistent with an arrest in mitosis (Figure 2C and Supplementary Figure 2B). We concluded that depletion of *ZBTB4* does not generally render cells resistant to vincristine, but selectively alters the response to low doses of the drug in the range predicated to activate p53.

Zbtb4 influences p53-mediated p21Cip1 expression

To understand how Zbtb4 shifts the response to vincristine from apoptosis to cell cycle arrest, we analysed how depletion of Zbtb4 affects the expression of a number of p53 target genes including *P21CIP1*, *NOXA* and *PUMA* (Figure 3B). There was no difference in the expression of the pro-apoptotic *BAX*, *PUMA* and *NOXA* between Zbtb4-depleted and control cells, either before treatment with vincristine (Figure 3B) or in response to treatment with the drug (not shown); similarly, the expression of two target genes of p53 that have been implicated in regulation of cell cycle progression, *14-3-3σ* and *BTG2*, was not consistently upregulated by depletion of Zbtb4 (Figure 3B). In contrast, both transient depletion of Zbtb4 using soluble siRNA (Figure 3B) and stable depletion using shRNAs (Figure 3C) led to an increase in expression of *P21CIP1* mRNA and p21Cip1 protein, both in untreated cells and after addition of vincristine (Supplementary Figure 3A). Conversely, stable expression of Zbtb4 from a retroviral vector reduced expression levels of *P21CIP1* mRNA, but had little or no effect on the levels of *PUMA* or *NOXA* mRNAs (Figure 3D and Supplementary Figure 3B). Furthermore, a microarray analysis of cells depleted of Zbtb4 confirmed that induction of *P21CIP1* mRNA was the strongest change in gene expression observed in such cells (Supplementary Figure 4). Taken together, we concluded that Zbtb4 represses transcription of *P21CIP1* both in unstressed cells and in response to vincristine.

To determine whether the enhanced expression levels of *P21CIP1* found in Zbtb4-depleted cells are causal for the resistance to vincristine, we depleted p21Cip1 using stable expression of shRNA in several clones of Zbtb4-depleted SH-EP cells (Figure 3E and Supplementary Figure 3C). Cells were exposed to vincristine and cell survival was measured after 72 h. In these experiments, three independent vectors targeting *P21CIP1* led to a moderate reduction in survival of control cells, and almost completely abolished the survival advantage of Zbtb4-depleted cells in the presence of vincristine. We concluded that depletion of Zbtb4 enhances survival

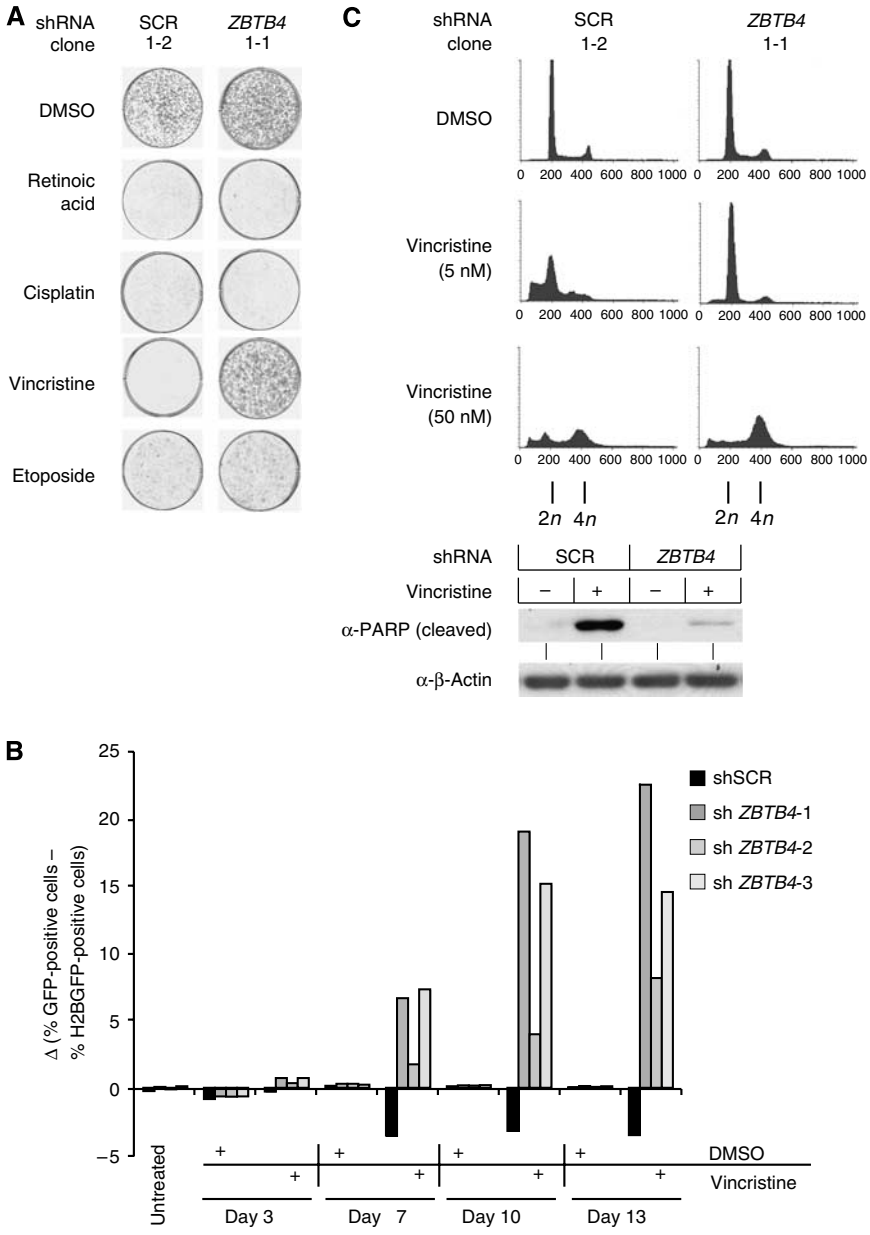


Figure 2 Depletion of Zbtb4 enables neuroblastoma cells to survive in the presence of vincristine. (**A**) Colony assays documenting growth of a control clone of SH-EP cells and a clone expressing the indicated sh*ZBTB4* RNA after treatment with the indicated chemotherapeutic agents. Control (DMSO treated) plates were stained at day 7 and experimental plates stained 14 days after plating. Note that the *ZBTB4* mRNA expression level of this clone is documented in Supplementary Figure 1A. (**B**) Cells expressing sh*ZBTB4* have a survival advantage in the presence of vincristine. SH-EP cells were infected with vectors that express GFP and either a control shRNA vector or one of three distinct sh*ZBTB4* RNAs as indicated. At the indicated time points after infection, the percentage of GFP-positive cells was measured by FACScan. All values were normalized to those obtained from a control plate expressing H2B-GFP. At the start of the experiment, approximately 10% of all cells were positive for GFP. (**C**) Vincristine induces apoptosis in control cells but G1 arrest in Zbtb4-depleted cells. The upper panels document FACS analyses of the DNA content of either a control clone or of a clone expressing sh*ZBTB4* after treatment with DMSO or the indicated concentrations of vincristine for 48 h. The lower panels show immunoblots using antibodies directed against cleaved poly-ADP-ribose-polymerase and β-actin as loading control.

of neuroblastoma cells in response to vincristine at least in part through upregulation of *P21CIP1*.

Induction of p21Cip1 mediates cell cycle arrest downstream of p53; to analyse whether Zbtb4 affects this arrest, we infected SH-EP neuroblastoma cells with control or retroviruses expressing HA-tagged Zbtb4 and treated both cell pools with nutlin, a small molecule inhibitor of the Hdm2 ubiquitin ligase (Vassilev *et al*, 2004). Immunoblots showed that p53 accumulated to a similar extent in both cell pools (Figure 4A). p21Cip1 accumulated in response to nutlin in control cells, but this induction was suppressed in cells expressing ectopic Zbtb4. FACScan analysis showed that SH-EP cells accumulated in the G1 phase of the cell cycle in response to exposure to nutlin for 33 h (Figure 4B); under these conditions, exposure to nutlin did not induce apoptosis (Figure 4B) and elicited only small increases in the levels of *PUMA* and *NOXA* mRNAs (not shown). In contrast to control cells, cells expressing Zbtb4 showed no change in the cell

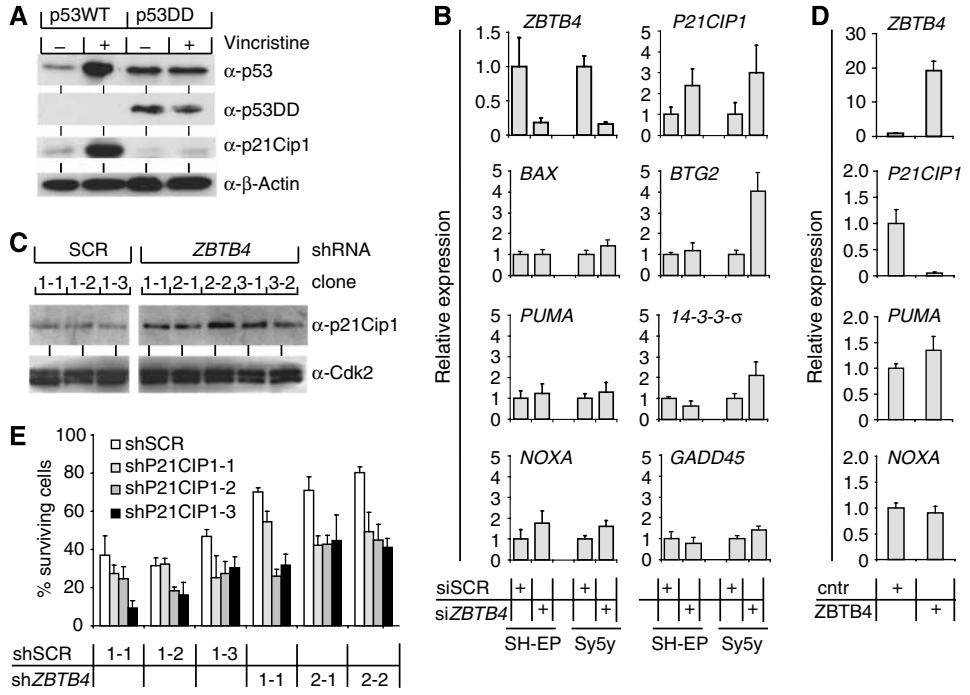


Figure 3 Depletion of Zbtb4 causes resistance to vincristine treatment through upregulation of *P21CIP1*. (**A**) Vincristine induces activation of p53. The panels show immunoblots of SH-EP cells infected with either a control vector (left) or a vector expressing a dominant-negative allele of p53 (p53DD; right) probed with the indicated antibodies. (**B**) Transient depletion of Zbtb4 upregulates *P21CIP1* mRNA levels. The panels document the RQ–PCR analysis of relative mRNA expression level of *ZBTB4*, *P21CIP1*, *14-3-3σ*, *BTG2*, *GADD45*, *PUMA*, *BAX* and *NOXA* in SH-EP and SH-SY5Y neuroblastoma cells transfected with either control or siRNA targeting *ZBTB4*. Cells were harvested 48 h after transfection and expression levels were determined by RQ–PCR. (**C**) Stable depletion of Zbtb4 upregulates p21Cip1 protein. The panels show immunoblots of p21Cip1 and Cdk2 protein levels in indicated SH-EP cell clones stably infected with either control vectors or vectors expressing sh*ZBTB4*. (**D**) Ectopic expression of Zbtb4 represses *P21CIP1*. The panels document the RQ–PCR analysis of the relative mRNA expression level of *ZBTB4*, *P21CIP1*, *PUMA* and *NOXA* in SH-EP cells stably infected with HA-tagged *ZBTB4* vector or a control vector. (**E**) *P21CIP1* is required for protection of SH-EP cells from vincristine-induced apoptosis by depletion of Zbtb4. The indicated SH-EP cell clones stably expressing either sh*ZBTB4* or control shRNA were transiently transfected with vectors expressing sh*P21CIP1* or a scrambled shRNA. At 48 h after transfection, cells were plated in triplicate for vincristine treatment. Cells were treated with either vincristine or DMSO for 3 days; subsequently, the relative cell number was determined. Error bars represent standard error of the mean.

cycle distribution in response to nutlin treatment (Figure 4B). Identical results were obtained after 50 h of treatment, after which the cells became confluent (not shown). Retroviral expression of *P21CIP1* partially restored G1 arrest in cells expressing Zbtb4 in response to nutlin, arguing that suppression of *P21CIP1* causally contributes to the failure of Zbtb4-expressing cells to arrest in response to nutlin (not shown).

We wondered how Zbtb4 affected the cellular response to DNA-damaging agents and exposed SH-EP cells to adriamycin, cisplatin or etoposide in addition to vincristine and nutlin. At the concentrations used, all drugs led to an accumulation of p53 and p21Cip1 and ectopic expression of Zbtb4 repressed this increase in p21Cip1 levels in response to all drugs (Figure 4C). Similar to the responses to nutlin, expression of Zbtb4 reduced the percentage of cells arrested in the G1 phase of the cell cycle in response to all drugs (shown for adriamycin and etoposide in Figure 4D). In contrast to treatment with nutlin, cells treated with DNA-damaging agents accumulated phosphorylated p53 and Chk1, indicative of activation of the ATM and/or ATR kinases (Figure 4C); this correlated with an arrest in the G2 phase in addition to the G1 arrest (Figure 4D). Zbtb4 did not alleviate the G2 arrest in response to these drugs. Our findings are consistent with multiple data demonstrating that cells arrest in the G2 phase independently of p21Cip1 upon exposure to DNA damage (Hirao *et al*, 2000). Taken together, the data show that Zbtb4 specifically suppresses arrest in the G1 phase in response to activation of p53.

Zbtb4 binds to the P21CIP1 promoter

We considered two models on how Zbtb4 might repress expression of *P21CIP1*. First, we tested the hypothesis that Zbtb4 might exert an effect upstream of p53. To address this possibility, we generated clones and pools of SH-EP cells that express a carboxyl-terminal fragment of p53 (p53DD) that exerts an effect as a dominant-negative allele as it sequesters p53 into non-functional heterotetramers (Shaulian *et al*, 1992). As expected, p53DD impaired the ability of SH-EP cells to upregulate *P21CIP1*, *NOXA* and *PUMA* in response to both UV irradiation and to vincristine (Figures 3A and 5A). In contrast, p53DD did not abolish the effect of shRNA targeting Zbtb4 on *P21CIP1* expression in SH-EP cells, arguing that Zbtb4 does not exert an effect upstream of p53 (Figure 5B). To confirm this result, we repeated the experiment in HCT116 colon carcinoma cells and in a clone of these cells, in which both alleles of *P53* have been disrupted. Depletion of Zbtb4 induced expression of *P21CIP1* in both cell types (Figure 5B). We concluded that the effects of Zbtb4 on *P21CIP1* expression are independent of p53.

We then tested the hypothesis that Zbtb4 might directly regulate the activity of the *P21CIP1* promoter. Zbtb4 potently repressed transcription when tethered to DNA through a

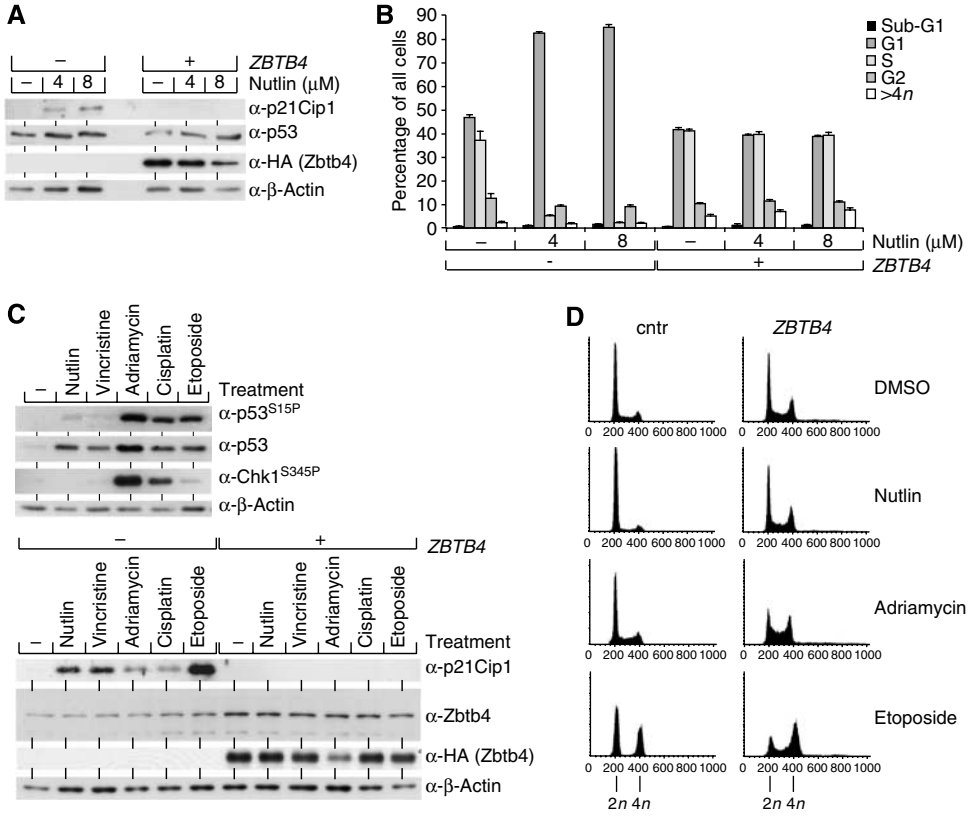


Figure 4 Zbtb4 suppresses cell cycle arrest in response to activation of p53. (**A**) Zbtb4 represses p21Cip1 expression in response to nutlin treatment. SH-EP cells were stably infected with either control retroviruses or viruses expressing HA-tagged Zbtb4. Pools of infected cells were either treated with DMSO as solvent control or with the indicated concentrations of nutlin for 33 h. The panels show immunoblots of cell lysates probed with the indicated antibodies; β-actin served as a loading control. (**B**) FACScan analysis showing the response to nutlin. The panel documents the cell cycle distribution of either control cells or SH-EP cells expressing Zbtb4 after treatment with the indicated concentrations of nutlin for 33 h. Error bars represent the standard deviation of triplicates. (**C**) The upper panels show immunoblots documenting expression of p53, phosphorylated p53 ($p53^{S15P}$) and of phosphorylated Chk1 ($Chk1^{S345P}$) in SH-EP cells treated with the indicated agents. The lower panels show immunoblots documenting expression of p21Cip1, total Zbtb4 and of exogenous Zbtb4 (detected using a HA antibody) in control SH-EP cells and in SH-EP cells expressing Zbtb4; β-actin was used as a loading control. (**D**) FACScan analysis of the responses to nutlin, adriamycin and etoposide of control SH-EP cells and of SH-EP cells expressing Zbtb4.

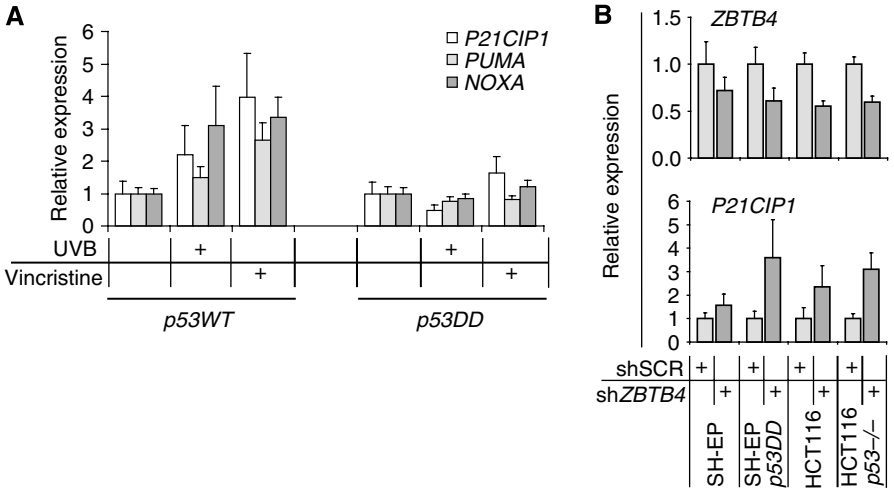


Figure 5 Zbtb4 regulates *P21CIP1* expression independently of p53. (**A**) Expression of a dominant-negative allele of p53 blocks UV- and vincristine-induced upregulation of *P21CIP1*, *NOXA* and *PUMA*. Exponentially growing SH-EP cells were stably infected either with control retroviral vectors or with vectors expressing a carboxyl-terminal fragment of p53 (p53DD). Pools of infected cells were exposed to UVB or treated with 5 nM vincristine or DMSO as a control for 24 h. Cells were harvested and expression of the indicated genes was analysed by the RQ–PCR as before. (**B**) Regulation of *P21CIP1* by Zbtb4 is independent of p53. Control SH-EP cells, SH-EP p53DD-transduced cells, HCT116 cells and HCT116 $p53^{-/-}$ cells were transfected with si*ZBTB4* or control (scrambled) siRNA. At 48 h after transfection, cells were harvested and the expression of *ZBTB4* and *P21CIP1* was determined by the RQ–PCR analysis. Data are plotted as expression relative to an internal standard.

Gal4-DNA-binding domain (not shown), consistent with previous observations (Filion *et al*, 2006). The *P21CIP1* promoter contains two consensus-binding sites for Zbtb4 (which are identical to the non-methylated Kaiso-binding sites; see Discussion) at −380 and −195 relative to the start site of transcription (Figure 6A). Transient transfection experiments using several reporter plasmids that contain different fragments of the *P21CIP1* promoter showed that Zbtb4 had little effect on basal *P21CIP1* promoter activity in these assays (Figure 6B). To mimic activation of *P21CIP1* in response to DNA damage, we expressed the transcriptional activator Miz1, which is required for DNA damage-induced activation of *P21CIP1* expression (Wanzel *et al*, 2005). Zbtb4 inhibited activation of *P21CIP1* by Miz1. Chromatin immunoprecipitation (ChIP) experiments showed that Zbtb4 binds to the core promoter of the *P21CIP1* gene *in vivo*, but not to a control region located in the 3-untranslated region (3′UTR); shRNA-mediated depletion of Zbtb4 abolished the signal observed in ChIP experiments, documenting the specificity of the antibody (Figure 6C). Taken together, the data show that Zbtb4 binds to the *P21CIP1* promoter *in vivo* and represses its activity.

Surprisingly, Zbtb4 also repressed transcription from promoter fragments spanning from −144 to +16 and from −96 to +16 nucleotides relative to the transcription start site that do not contain any candidate Zbtb4-binding sites (Figure 6A and B). As Zbtb4 can bind to methylated DNA independent of its binding site, we considered the possibility that repression of *P21CIP1* by Zbtb4 reflects methylation of the *P21CIP1* promoter; however, treatment of cells expressing Zbtb4 with the demethylating agent, azacytidine, had no effect on repression of p21Cip1 expression, rendering this possibility unlikely (Supplementary Figure 5). The core *P21CIP1* promoter contains a binding site for the transcription factor Miz1 and the reporter assays described above showed that Zbtb4 repressed Miz1-driven transcription of the *P21CIP1* promoter without affecting the expression of Miz1 protein (Figure 6B and data not shown). Furthermore, ChIPs showed a strong signal for binding of Zbtb4 not only around its cognate-binding site but also at the transcription start site (Figure 6C and D). Although the binding sites are so close that the two primer pairs used to amplify the immunoprecipitated DNA do not completely discriminate between both binding sites (see the low amount of Miz1 binding detected using the primers spanning the Zbtb4-binding sites), the strong signal observed for Zbtb4 around the start site cannot be accounted for by co-amplification of DNA immunoprecipitated through Zbtb4 bound to its cognate-binding site (Figure 6D).

As POZ domain transcription factors can form heterodimers, we wondered whether Zbtb4 heterodimerizes with Miz1. To address this question, we expressed both Miz1- and HA-tagged Zbtb4 by transient transfection in HEK293 cells; in these experiments, α-HA antibodies efficiently co-precipitated Miz1 and vice versa, α-Miz1 antibodies co-precipitated Zbtb4 (Figure 6E). Importantly, α-HA antibodies did not precipitate Miz1 when Zbtb4 was not expressed (Figure 6E). Furthermore, neither monoclonal nor polyclonal control antibodies precipitated Miz1 or Zbtb4 (Figure 6E). We repeated the experiment in the presence of high concentrations of ethidium bromide to exclude the possibility that the co-precipitation was mediated by binding of both proteins to DNA; this did not affect co-immunoprecipitation, demonstrating that both proteins interact independently of their ability to bind DNA (Figure 6E). Consistent with the previous finding that Miz1 interacts with Myc, precipitation of Zbtb4 also co-immunoprecipitated Myc when both proteins were co-expressed by transient transfection; in contrast, Zbtb4 bound less efficiently to MycV394D, a point mutant of Myc that does not bind to Miz1, suggesting that Zbtb4 binds to Myc indirectly through Miz1 (Supplementary Figure 6). To test whether endogenous Zbtb4 interacts with Miz1, we performed immunoprecipitations from colon carcinoma (LS174T) cells, which express high levels of endogenous Miz1 (Figure 6F). Antibodies directed against Zbtb4, but not control antibodies co-precipitated endogenous Miz1 in these experiments, demonstrating that endogenous Miz1 and Zbtb4 interact *in vivo*. Oligonucleotide pulldown experiments showed that both Miz1 and Zbtb4 bound to oligonucleotides spanning either the Zbtb4- or the Miz1-binding sites of the *P21CIP1* promoter, but not to a control oligonucleotide (Figure 6G). Furthermore, Zbtb4 enhanced DNA binding of Miz1 to either site. To test whether Miz1 recruits Zbtb4 to its cognate-binding site *in vivo*, we repeated the ChIP in cells transfected with either scrambled siRNA or siRNA targeting Miz1; depletion of Miz1 reduced binding of Zbtb4 to the *P21CIP1* promoter (Figure 6H). Taken together, we concluded that Zbtb4 and Miz1 form a DNA-binding complex on the *P21CIP1* promoter in human cells.

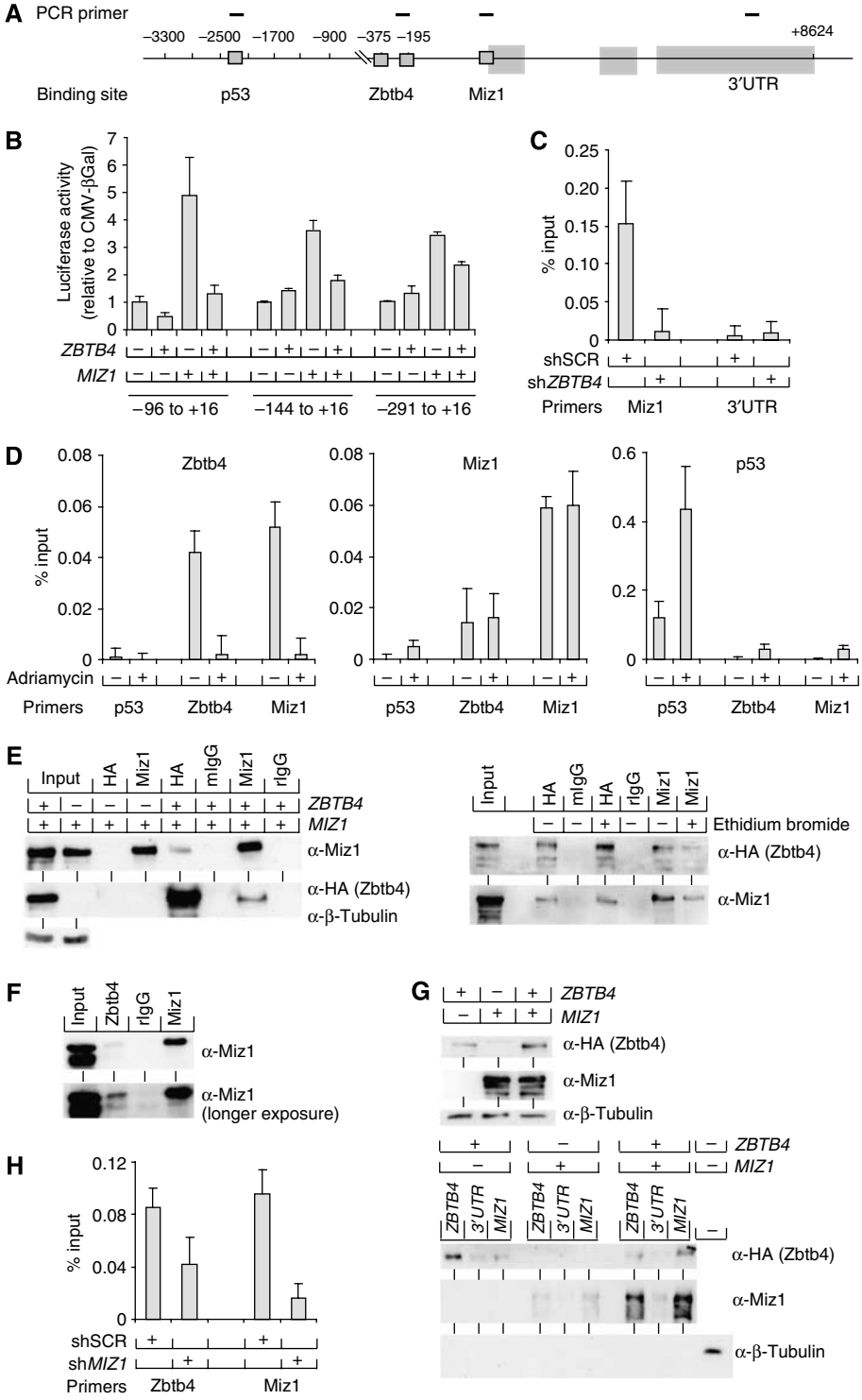
These data suggest a model in which Zbtb4 represses expression of *P21CIP1* in unstressed cells but that repression is relieved when *P21CIP1* is activated. To test this model, we repeated the ChIPs in cells exposed to the DNA-damaging agent, adriamycin. Exposure to DNA damage induced binding of p53 to its cognate-binding site on the *P21CIP1* promoter; at the same time, binding of endogenous Zbtb4 to the *P21CIP1* promoter was reduced, whereas binding of Miz1 was not affected by DNA damage (Figure 6D). Taken together, these findings support a model in which release of Zbtb4-mediated repression contributes to the activation of the *P21CIP1* promoter in response to DNA damage.

Zbtb4 recruits Sin3a and Sin3b

To understand how Zbtb4 represses *P21CIP1* expression, we monitored histone modifications at the *P21CIP1* promoter in control cells and in cells expressing Zbtb4 using ChIP (Figure 7A). Expression of Zbtb4 led to a decrease in histone H3 acetylation at the *P21CIP1* promoter, suggesting that Zbtb4 recruits a histone deacetylase (HDAC). In contrast, only low levels of histone H3K9 and histone H3K27 methylation, additional modifications that are associated with transcriptional repression, were detected and these were unaffected by Zbtb4. Zbtb4 also induced a decrease in H4K20 methylation, a mark that has been associated with transcriptional elongation. Consistently, the signal was high close to the transcription start site and in the 3′UTR of the *P21CIP1* locus (Figure 7A). To test whether histone deacetylation is critical for Zbtb4-mediated repression, we treated SH-EP cells with two inhibitors of HDACs, trichostatin-A and sodium butyrate. Both inhibitors significantly reduced repression of *P21CIP1*, suggesting that Zbtb4-mediated histone deacetylation mediates repression by Zbtb4 (Figure 7B).

The POZ domain protein Bcl-6 recruits HDAC complexes through binding to Sin3a (Dhordain *et al*, 1998). We therefore

determined whether Zbtb4 associates with Sin3a and/or Sin3b. Immunoprecipitation of ectopically expressed HA-tagged Zbtb4 co-precipitated endogenous Sin3a from extracts of HEK293 cells (Figure 7C). Furthermore, immunoprecipitation of endogenous Zbtb4 co-precipitated both endogenous Sin3a and Sin3b from HCT116 cell lysates (Figure 7D). Consistent with this observation, immunoprecipitation of exogenous Zbtb4 precipitated significant HDAC activity (Figure 7E). Moreover, shRNA-mediated depletion of the Sin3 complex subunit Sds3, which is required for HDAC binding, results in reduced recruitment of HDAC activity by Zbtb4, indicating that recruitment of HDAC activity by Zbtb4 is dependent on Sin3a or Sin3b (Figure 7E and F). Parallel experiments showed that Zbtb4 co-immunoprecipitates HDAC2, one of the HDACs bound by Sin3, from lysates of transfected cells (not shown). ChIP experiments confirmed that Zbtb4 recruits both Sin3a and Sin3b to the start site of the *P21CIP1* promoter, but not to the 3′UTR (Figure 7G).



To demonstrate the functional relevance of this interaction, we carried out expression profiling of HCT116 cells treated with shRNAs to either deplete Zbtb4 or Sin3a and Sin3b (Figure 7H). We identified 192 genes that were upregulated in response to depletion of Zbtb4 and 265 genes that were upregulated in response to co-depletion of Sin3a and Sin3b. The analysis identified 29 genes, among them *P21CIP1*, that were upregulated in response to both Sin3a/b and Zbtb4 depletion. Taken together, our data show that a Zbtb4/Miz1/Sin3 complex represses *P21CIP1* in response to p53 activation and suggests that there is a group of genes that may be similarly repressed by a Zbtb4/Sin3 complex.

Discussion

The POZ domain zinc-finger protein Zbtb4 has been identified based on its homology to the Kaiso protein (Filion *et al*, 2006); Kaiso is a transcriptional repressor protein that binds to methylated DNA sequences in a sequence-independent manner and to non-methylated DNA that contains a Kaiso binding site (KBS) (Daniel *et al*, 2002). Both Zbtb4 and Kaiso are POZ/BTB domain zinc-finger proteins; however, close sequence similarity between both proteins is restricted to the zinc-finger DNA-binding domain. Consequently, Zbtb4 shares with Kaiso the ability to bind both methylated DNA and the KBS (Filion *et al*, 2006). In addition, both Zbtb4 and Kaiso are transcriptional repressor proteins.

We now identify the *P21CIP1* gene, which encodes an inhibitor of the cyclin-dependent kinase Cdk2, as a target for transcriptional repression by Zbtb4. p21Cip1 mediates cell cycle arrest in response to several antimitogenic signals, including activation of p53. Consistent with this notion, Zbtb4 regulates cell cycle arrest under conditions in which p53 is activated by vincristine, which facilitates the nuclear import of p53, or nutlin, which inhibits the Hdm2 E3 ligase that degrades p53 (Giannakakou *et al*, 2000; Vassilev *et al*, 2004). Zbtb4 does not affect the response to genotoxic agents in clonogenic assays and this correlated with its inability to affect the arrest in S and G2 phases in response to such agents. Taken together, our data show that Zbtb4 has little function in regulating the proliferation of unstressed cells but promotes progression through the G1 phase in cells in which p53 is activated.

Paradoxically, however, our interest in Zbtb4 was prompted by the observation that its expression is downregulated in advanced stages of multiple solid tumours, resulting in upregulation of *P21CIP1* expression. Indeed, elevated levels of p21Cip1 are found in several advanced human tumours, suggesting that p21Cip1 can provide a selective advantage during tumorigenesis (Erber *et al*, 1997). Furthermore, *p21cip1*-deficient mice are less susceptible than wild-type mice to Myc-induced mammary tumorigenesis (Bearss *et al*, 2002) and to lymphomagenesis that occurs in *atm*-deficient mice (Wang *et al*, 1997).

Depletion of Zbtb4 suppresses the normal apoptotic response upon vincristine-mediated activation of p53. We show that induction of *P21CIP1* is essential for this suppression. This is consistent with previous observations showing that p21Cip1 can provide critical antiapoptotic signals to cells. Biochemically, this may be mediated indirectly through inhibition of Cdk2 by p21Cip1, as activation of cyclin A/cdk2 complexes can be a critical step in apoptosis (Levkau *et al*, 1998). Alternatively, p21Cip1 inhibits the pro-apoptotic kinases Ask1 and SAPK(JNK) and the cleavage and activation of pro-caspase 3; therefore, the antiapoptotic functions of p21Cip1 may be independent of its ability to regulate Cdk2 activity (Zhan *et al*, 2007).

Importantly, p21Cip1 and Zbtb4 may have critical oncogenic functions independent of the p53 status of a cell. For example, p21Cip1 protects epithelial cells from anoikis by suppressing the expression of the pro-apoptotic BH3-only protein Bim (Collins *et al*, 2005). Anoikis inhibits metastasis, as it eliminates epithelial cells that have detached from the basal membrane and have lost cell–substratum contact. Notably, expression of *ZBTB4* discriminates metastatic from non-metastatic breast, prostate and lung tumours (Supplementary Table 2) and is found in the pan-tumour expression signature that predicts metastasis ($P = 0.003$) (Ramaswamy *et al*, 2003). Downregulation of Zbtb4 may therefore facilitate the escape of tumour cells from anoikis. Furthermore, p21Cip1 has been implicated in maintaining

Figure 6 Zbtb4 and Miz1 form a transcriptional repressor complex that represses transcription of *P21CIP1*. (**A**) Scheme of the human *P21CIP1* gene with indicated p53, Miz1 and putative Zbtb4-binding sites. Also indicated are the positions of primers used for the chromatin immunoprecipitation (ChIP) experiments shown below. Shaded grey areas denote exons. (**B**) Zbtb4 represses *P21CIP1* promoter activity. Shown are reporter assays using *P21CIP1* promoter constructs spanning the indicated nucleotides relative to the transcription start site. The graph shows the luciferase activity relative to the standard CMV-βGal after transfection of SH-EP cells with either expression plasmids encoding Zbtb4, Miz1 or both as indicated. The error bars represent the standard deviation of triplicate samples. Controls established that Zbtb4 does not inhibit expression of Miz1 (not shown). (**C**) ChIP demonstrating binding of Zbtb4 to the core of the *P21CIP1* promoter, but not to a control region located in the 3′UTR. Cells were infected with viruses expressing either control shRNA or sh*ZBTB4* as indicated. (**D**) Activation of *P21CIP1* correlates with loss of endogenous Zbtb4 from the *P21CIP1* promoter. The panels show ChIP assays documenting binding of p53, Miz1 and Zbtb4 to the *P21CIP1* promoter in untreated SH-EP cells and in SH-EP cells exposed to adriamycin. For each protein, the difference between specific antibody and control antibody in percentage DNA bound is plotted. (**E**) Zbtb4 binds to Miz1 in lysates of co-transfected cells. HEK293 cells were transfected with expression vectors encoding HA-tagged Zbtb4 and/or Miz1 as indicated. Lysates were immunoprecipitated with the indicated antibodies. The left panels show immunoblots of the immunoprecipitates probed with the indicated antibodies. The right panels document an identical experiment except that 0.1 mg/ml ethidium bromide was added to the lysates before immunoprecipitation to exclude that the observed interaction is mediated by binding of both proteins to DNA. Both antibodies IgG mouse and IgG rabbit were used as controls. (**F**) Endogenous Zbtb4 and Miz1 co-immunoprecipitate in LS174T cells. Rabbit IgG was used as a control. (**G**) Zbtb4 and Miz1 form a DNA-binding complex. HEK293 cells were transfected with expression plasmids encoding Miz1 and/or Zbtb4 as indicated. Biotinylated oligonucleotides spanning the Zbtb4-binding site, the Miz1-binding site or a control sequence (3′UTR) were added and precipitated using avidin-sepharose. Bound proteins were detected by immunoblotting using the indicated antibodies. (**H**) Miz1 recruits Zbtb4 to the core *P21CIP1* promoter. SH-EP cells were transfected either with scrambled siRNA or siRNA targeting *MIZ1*. ChIP assays were performed with control and Zbtb4 antibodies and analysed using the indicated primer pairs. The panel shows the difference between the specific signal and the signal obtained with the control antibody.

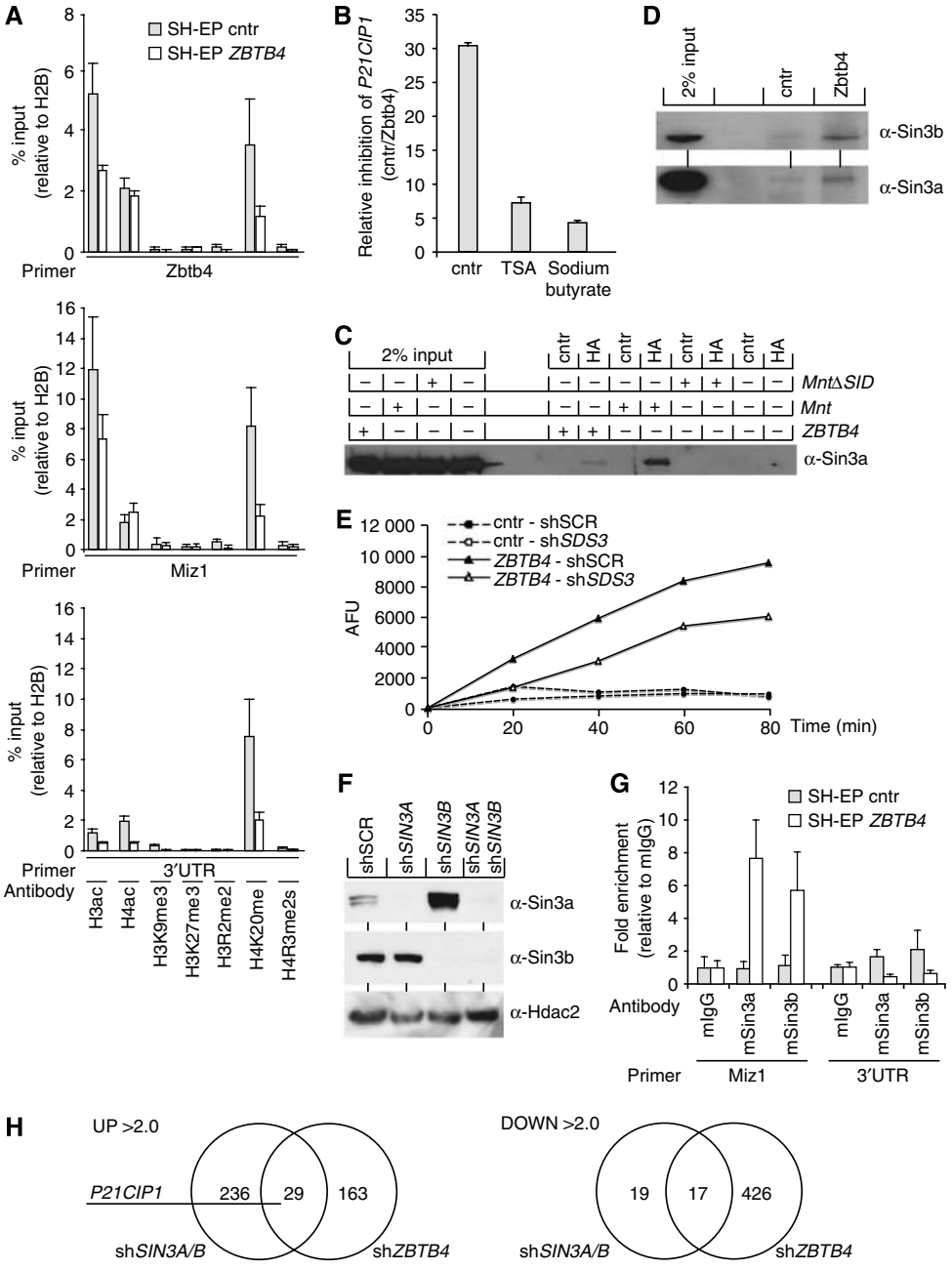


Figure 7 Zbtb4 recruits Sin3a to repress the *P21CIP1* promoter. (**A**) ChIP assays documenting Zbtb4-induced alterations in histone modification at the *P21CIP1* promoter. The assays were performed from either control SH-EP cells or SH-EP cells expressing Zbtb4 using the indicated antibodies. The signals were normalized to precipitations using a H2B antibody. (**B**) Inhibition of histone deacetylation reduces repression of *P21CIP1* by Zbtb4. SH-EP cells were infected with either control retroviruses or retroviruses expressing ectopic Zbtb4. Cell pools were either treated with solvent control or with the histone deacetylase inhibitors trichostatin-A (TSA) or sodium butyrate as indicated. Shown are expression data for *P21CIP1* mRNA relative to a control mRNA derived from the RQ–PCR analysis with error bars representing the standard deviation of the mean. The data are plotted as fold repression by Zbtb4 in each experimental condition. The concentration of TSA used was efficient in inducing overall histone acetylation as determined by immunoblot analysis (not shown). (**C**) Zbtb4 co-immunoprecipitates Sin3a. HEK293 cells were transfected with indicated HA-tagged vectors, and protein lysates were immunoprecipitated with HA antibodies. Associated endogenous Sin3a was visualized by immunoblotting. Here, 2% input of Sin3a is indicated. As a positive control, we used the repressor protein Mnt and a mutated version of Mnt, in which the Sin3a interaction domain had been deleted (MntΔSID) (Hurlin *et al*, 1997). (**D**) Endogenous Zbtb4 co-immunoprecipitates Sin3a and Sin3b. HCT116 whole cell extracts were immunoprecipitated with Zbtb4 or pre-immune antibodies; precipitates were probed with antibodies to mSin3b and mSin3a. Here, 2% of input is shown. (**E**) Association of Zbtb4 with histone deacetylase activity depends on Sds3. HEK293 cells were transfected with constructs expressing Zbtb4 or control vector and simultaneously infected with lentiviruses expressing either control shRNA or shRNA targeting Sds3. At 48 h later, whole cell lysates were prepared and immunoprecipitated with Zbtb4 or normal rabbit serum antibodies. HDAC activity in the immunoprecipitates was analysed. (**F**) Characterization of lentiviral shRNAs targeting *SIN3A* and *SIN3B*. HCT116 cells were infected with lentiviruses expressing the indicated shRNAs. At 48 h after infection, cells were harvested. Immunoblotting was performed with the indicated antibodies. (**G**) Zbtb4 recruits Sin3a and Sin3b to the core *P21CIP1* promoter. Shown are ChIP assays from control SH-EP cells and from SH-EP cells expressing Zbtb4. (**H**) Zbtb4 shares transcriptional targets with Sin3. Comparison of transcripts regulated in Sin3- and Zbtb4-depleted cells relative to control HCT116 cells. Absolute values of the fold changes are indicated as >2.0-fold. The probability of chance of the overlap between sh*SIN3* and sh*ZBTB4* as calculated by the hypogeometric distribution is $<10^{-4}$.

stem cell pools and suppression of Zbtb4 expression may have a critical role in maintaining quiescence of tumour-initiating cells.

Zbtb4 recruits an mSin3/HDAC complex that is likely to be involved in repression of *P21CIP1* expression. Several HDACs, notably HDAC1, 2 and 3, repress *P21CIP1* in different cell types; therefore, expression of *P21CIP1* is an important target that mediates cell cycle arrest in response to pharmacological inhibition of histone deacetylation (Lagger *et al*, 2003). Upon transient overexpression, Sp1 and Sp3, which bind to the core *P21CIP1* promoter, bind to HDACs and recruit them to the *P21CIP1* gene (Lagger *et al*, 2003). It is less clear whether endogenous Sp proteins recruit HDACs to the *P21CIP1* promoter. In contrast, POZ domain complexes function as dedicated repressor proteins and the Miz1/Zbtb4 complex is a potent repressor of *P21CIP1* promoter activity. In this view, we suggest that this complex may be a key target for cell cycle arrest by inhibition of HDAC activity and levels of Zbtb4 may have prognostic value in determining the response to such drugs.

Materials and methods

Patients

We studied tumour specimens from 98 children with primary neuroblastoma who had been diagnosed from 1981 to 2000. All patients were treated according to previously described protocols with confirmed consent for study procedures.

Cell culture

HEK293 and HeLa cells were grown in DMEM, HCT116 were grown in McCoy's, Ls174T and SH-EP cells were grown in RPMI medium. Where indicated, cells were treated with 4 μM nutlin-3 (Sigma), trichostatin-A (50 ng/ml), 2 and 50 nM vincristine, 40 μM all-*trans* retinoic acid, 0.2 μM etoposide, 0.05 μM cisplatin, 5 mM sodium butyrate or 300 nM azacytidine. UV irradiation experiments used 250 J/m^2 UVB light. Soluble siRNA against *ZBTB4* (Dharmacon) and scrambled were transfected with lipofectamin RNAimax (Invitrogen).

A full-length cDNA encoding HA-tagged Zbtb4 was assembled from PCR-generated fragments, sequenced and inserted into the pCMV and pBabe-puro vectors. Three shRNAs targeting *ZBTB4* and scrambled controls were cloned into pRetroSuper-puro and pRetroSuper-GFP, respectively. shRNAs directed against *P21CIP1* were cloned into pSuper Neo-GFP. ShRNAs directed against *SIN3a*, *SIN3b*, *ZBTB4* and control were cloned into a lentiviral vector derived from the FUGW backbone (courtesy of Valeri Vasioukhin—pLenti-H1). The shRNA sequences are available upon request.

Antibodies

The following antibodies were used: α-HA (HA.11; Covance), α-p21Cip1 (N-20), α-p53 (DO-1), α-Cdk2 (M2), α-mSin3b (H4) (all Santa Cruz Biotechnology), α-β-tubulin (MAB3408; Chemicon), α-Miz1 (10E2, Herold *et al*, 2002; H190, Santa Cruz Biotechnology) and α-Zbtb4 (Filion *et al*, 2006).

Reporter assays and ChIP

The p21Cip1-promoter-luciferase reporter assays have been described in Wu *et al* (2003). ChIP assays were performed as described previously (Bouchard *et al*, 2001) using primers specific for the Miz1-binding site (−32 to +47 relative to the start site of transcription), the promoter-proximal Zbtb4 binding site (−276 to −171), p53-binding site (−2289 to −2164) and a sequence located in the 3′UTR of the gene (+7464 to +7641) as a control. Standard deviations of triplicate were calculated according to the Gaussian error distribution law.

RNA extraction and RQ–PCR analysis

Real-time PCR analysis was performed in triplicate. Relative expression between samples and control was calculated according to the $\Delta\Delta C_T$ relative quantification method using β2-microglobulin as a standard. Primer sequences are available upon request.

HDAC activity

HDAC assays were performed using the Fluor de Lys HDAC assay kit as specified by the manufacturer (Biomol, Plymouth, PA).

Microarray analysis

Expression profiling studies were performed using Affymetrix GeneChip Human U133A 2.0 arrays. Sample labelling and hybridizations were performed with adherence to Affymetrix's standardized protocols. The data sets were subsequently grouped for pairwise analysis consisting of two groups of three biological replicates. The accession number is E-MEXP-1509.

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