HDAC3 is linked to cell cycle machinery in MiaPaCa2 cells by regulating transcription of *skp2*

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Abstract. *Objective:* Histone deacetylases (HDACs) have been linked to cell cycle control in various models, involving regulation of the cyclin-dependent kinase inhibitor p27^{Kip1}. *Results:* Here, we demonstrate that HDAC inhibition by trichostatin A reduces S-phase kinase-associated protein 2 mRNA and protein abundance. Furthermore, in contrast to HDAC1, recruited to the *skp2* promoter in the G₀ phase, HDAC3 is bound in early S phase. Activating function of HDAC3 towards the *skp2* gene has been validated using RNA interference techniques. siRNAs, targeting HDAC3 specifically, reduced *skp2* transcription. *Conclusion:* These findings propose that the *skp2* gene is a novel target of HDAC3, mediating cell cycle control and oncogenesis.

INTRODUCTION

Histone acetylases (HATs) and histone deacetylases (HDACs) are important enzymes that catalyse reversible acetylation of histone tails (Santos-Rosa & Caldas 2005). Recent evidence suggests that HDAC inhibitors, like trichostatin A (TsA), inhibit proliferation of tumour cells by inducing G₁-phase arrest (Kouzarides 1999). This arrest correlates with the up-regulation of mRNA and protein levels of p21^{WAF1} and p15^{INK4b} (Nakano *et al.* 1997; Hitomi *et al.* 2003). Although cyclin-dependent kinase inhibitor p27^{Kip1} protein increases in various model systems after HDAC inhibitor treatment and contributes to cell cycle regulation, molecular mechanisms have not yet been fully elucidated (Wharton *et al.* 2000; van de Mark *et al.* 2003; Chen & Faller 2005).

Even though p27^{Kip1} is also controlled at the level of its synthesis, protein abundance is mainly regulated by post-translational modifications, affecting p27^{Kip1} protein turnover. Phosphorylation of p27^{Kip1} on Thr-187 by cyclin E-cyclin-dependent kinase 2 complex tags the protein for recognition by S-phase kinase-associated protein 2 (*skp2*), an F-box protein that functions as a receptor component of SCF (*skp1*, *cull1*, F-box) ubiquitin ligase complex, resulting in p27^{Kip1} ubiquitylation and degradation (Nakayama & Nakayama 2005). Due to structural and biochemical characteristics, mammalian HDACs fall into three classes (Gray & Ekstrom 2001). Of mammalian

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class I enzymes (HDAC1, HDAC2, HDAC3, HDAC8 and HDAC11), HDAC1 and HDAC3 are linked to cell cycle control. HDAC1-deficient cells demonstrate impaired proliferation due to the transcriptional up-regulation of *p21^{WAF1}* and *p27^{Kip1}* (Lagger *et al.* 2002). HDAC3 promotes proliferation of HeLa and 293T cells and targets *p21^{WAF1}* and *p15^{INK4b}* gene promoters (Glaser *et al.* 2003; Huang *et al.* 2006).

In this study, we show that HDAC1 and HDAC3 are recruited to the *skp2* gene promoter. Whereas HDAC1 is bound to *skp2* promoter mainly in G₀, HDAC3 is recruited in early S phase. This implies that the activating function of HDAC3 towards the *skp2* gene may contribute to potential of HDAC3 to promote cell cycle progression.

MATERIALS AND METHODS

Reagents

Aphidicolin, cycloheximide (EMD Biosciences, San Diego, USA) and TsA (Biomol, Hamburg, Germany) were dissolved in dimethyl sulfoxide and stored at -20 °C.

Cell culture, transfection and siRNAs

MiaPaCa2 cells were cultured and siRNAs were transfected (200 nM) as recently described (Schneider *et al.* 2006b), siRNA sequences are available on request, these were purchased from Ambion, Austin, TX, USA.

Cell cycle synchronization

MiaPaCa2 cells were synchronized in G₀ phase by 48 h of serum starvation. Accumulation in early S phase was induced by 24 h aphidicolin (1 µg/mL) treatment.

Bromodeoxyuridine-incorporation assays

Bromodeoxyuridine (BrdU) incorporation assays were performed as described previously (Schneider *et al.* 2006b). Data were obtained from at least three independent experiments performed in duplicate and are presented as mean and standard error of the mean (SEM).

Protein analysis

Whole-cell lysates were prepared and Western blots were performed essentially as described previously (Schneider *et al.* 2006b). Antibodies to the following proteins were used: *p27^{Kip1}*, cyclin A (BD Pharmingen, San Diego, CA, USA), *skp2* (Zymed, San Francisco, CA, USA), cyclin E, HDAC3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin D1 (Oncogene Research Products, Bad Soden, Frankfurt, Germany), HDAC1 (upstate, Lake Placid, NY, USA) and β-actin (Sigma-Aldrich, Munich, Germany). Results of Western blots were quantified using Odyssey Infrared Imaging System (Licor, Bad Homburg, Hesse, Germany); data were obtained from at least three independent experiments.

Quantitative reverse-transcriptase PCR

Total RNA was isolated from the pancreatic carcinoma cell line MiaPaCa2 using the RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Quantitative mRNA was performed using real-time PCR analysis as previously described (Schneider *et al.* 2006b), and primer sequences are available on request. Data were obtained from at least three independent experiments performed in duplicate and are presented as mean and SEM.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assays were performed essentially as recently described (Schneider *et al.* 2006a). An equal amount of chromatin (50–100 µg) was used for each precipitation. Antibodies used were as follows: anti-RNA-Polymerase II, anti-HDAC3 (Santa Cruz Biotechnology), anti-acetyl-histone H3, anti-HDAC1 (Upstate) and anti-β-actin (Sigma). One-twentieth of precipitated chromatin was used for each PCR reaction. To ensure linearity, 28–38 cycles were performed, one representative result is shown. Bands were quantified using Quantity One 4.6 software (Bio-Rad Laboratories). One representative experiment of at least three performed is shown. Sequences of promoter-specific primers are: SKP2-95: 5'-CTCCCCGCCTACCCCGTGG-3'; SKP + 135: 5'-CAGACCCGCTAAGCCTAGCAACG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-FW: 5'-AGCTCAGGCCTCAAGACCTT-3'; GAPDH-RV: 5'-AAGAAGATGCGGCTGACTGT-3'; cyclin A-43: 5'-CAATAGTCGCGGGATACTTGAAGTGC-3' and cyclin A + 185: 5'-ACTCGCACCGACCCGGCCA-3'.

Plasmids and luciferase assay

Transfection of reporter genes (500 ng/well) were performed using FuGene6 (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol, in 12-well plates. For cotransfection, quantity of DNA was kept constant using empty pcDNA3 vector (Invitrogen, Karlsruhe, Germany). After indicated time points, cells were incubated in lysis buffer (Promega, Mannheim, Germany) for 15 min, were harvested and were cleared by centrifugation for 15 min. Lysates were normalized for protein content. Luciferase activity was determined in a LB 9501 luminometer (Berthold, Bad Wildbad, Germany) using a luciferase assay system (Promega). At least three independent transfection experiments were performed in triplicate. The 5'-regulatory region of the human *skp2* gene was amplified from human lymphocyte DNA by PCR using the following primers: SKP2-FW: 5'-CCCGCTCGAGCCCCCTTCCCTCTTCCACTGTTTTTC-3' and SKP2-RV: 5'-CCCGCTCGAGCCATTTTCATGCTCTTCCCCTTTTTGCAATC-3'. The resulting fragment was cloned into pCR4Blunt-TOPO (Invitrogen). SmaI digestion produces a 1217-bp fragment, which was blunt-ended cloned into pGL3basic (Promega), opened by SmaI digestion. This reporter gene construct was called *skp2-975/+224Luc* and was further digested with NheI/PfFI to *skp2-273/+244Luc* reporter gene construct. The HDAC3 expression plasmid was a kind gift of Dr E. Seto (Yang *et al.* 1997). pGL3control (Promega) was used as a control; all plasmids were verified by sequencing.

Statistical methods

All data were obtained from at least three independent experiments performed in duplicate, and the results are presented as mean and SEM. To demonstrate statistical significance Student's *t*-test was used in various experiments; *P*-values are indicated in figure legends.

RESULTS

Trichostatin A regulates *skp2* mRNA and protein abundance

Because p27^{Kip1} is known to contribute to the growth defect after HDAC inhibitor treatment, we investigated p27^{Kip1} abundance after TsA treatment in the pancreatic cancer cell line MiaPaCa2. As shown in Fig. 1a, we found distinct up-regulation of p27^{Kip1} protein expression 24 h after treatment of MiaPaCa2 cells with TsA. No change of p27^{Kip1} mRNA expression was found after 24 h of TsA treatment (Fig. 1b). Because p27^{Kip1} is regulated at the level of protein turnover, we

Figure 1. HDAC inhibition by Trichostatin A regulates *skp2* mRNA and protein abundance. Left figure (a): Western blot analysis of *skp2* and *p27^{Kip1}* expression 24 h after the treatment of MiaPaCa2 cells with 300 nM TSA. Middle figure: Quantification of *skp2* protein expression (Student's *t*-test: $*P = 0.02$ versus controls). Right figure: Quantification of *p27^{Kip1}* protein expression (Student's *t*-test: $**P = 0.004$ versus controls). (b) Real-time PCR analysis of *skp2* and *p27^{Kip1}* mRNA expression 24 h after the treatment of MiaPaCa2 cells with 300 nM TSA. *Skp2* and *p27^{Kip1}* mRNA was normalized to cyclophilin expression levels (Student's *t*-test: $*P = 0.002$ versus controls). (c) Western blot analysis of *p27^{Kip1}*. MiaPaCa2 cells were treated with 300 nM TSA (lanes 4–6) or were left as an untreated control. After 24 h, cells were treated with 50 μ g/mL cycloheximide for 2 h; Western blots show *p27^{Kip1}* degradation. β -actin detected on the same membrane controls loading and specificity.

tested for regulation of *skp2*, an important determinant of *p27^{Kip1}* stability (Nakayama & Nakayama 2005). Here, we found a decrease of *skp2* protein abundance after 24 h TSA treatment (Fig. 1a). Furthermore, a decrease of *skp2* mRNA to 24% compared to untreated controls was observed (Fig. 1b). Because *p27^{Kip1}* protein turnover is regulated *via* *skp2*, TSA treatment should lead to increased *p27^{Kip1}* protein stability. To examine this, we treated MiaPaCa2 cells with the protein synthesis inhibitor cycloheximide. A rapid decrease in *p27^{Kip1}* protein levels was observed, demonstrating high *p27^{Kip1}* turnover (Fig. 1c). In contrast, *p27^{Kip1}* protein levels were stabilized in our TSA-treated cells over the 2-h period investigated (Fig. 1c). These data suggest that transcriptional down-regulation of *skp2* after TSA treatment is responsible for increase of *p27^{Kip1}* protein abundance.

HDAC3 binds to the proximal *skp2* gene promoter early in S phase

To investigate *skp2* transcription in more detail, we synchronized our MiaPaCa2 cells in G_0 by serum starvation or in early S phase using aphidicolin, a reversible inhibitor of eukaryotic nuclear DNA replication. Figure 2a illustrates the synchronization using Western blot analysis. G_0 phase is characterized by lack of cyclin D1 expression, low *skp2* and high *p27^{Kip1}*, S phase is marked by increased *skp2*, cyclin D1, cyclin E and cyclin A expression and lack of *p27^{Kip1}* (Fig. 2a). To test for transcriptional up-regulation of *skp2* in S phase, we measured *skp2* mRNA levels and performed Chromatin immunoprecipitation assays. As shown in Fig. 2b, we observed

Figure 2. HDAC3 binds to the proximal *skp2* gene promoter in the early S phase. (a) MiaPaCa2 cells were synchronized in G₀ phase by 48 h of serum starvation, or in early S phase by using 1 µg/mL aphidicolin treatment for 24 h. Western blot analysis of cyclin D1, p27^{Kip1}, *skp2*, cyclin E, cyclin A, HDAC1 and HDAC3 expression. (b) MiaPaCa2 cells synchronized in the G₀ phase by 48 h of serum starvation or in early S phase using 1 µg/mL aphidicolin treatment for 24 h. mRNA abundance of *skp2* in G₀ phase and in S phase were quantified using real-time PCR analysis and were normalized to cyclophilin expression levels. (c) Chromatin of MiaPaCa2 cells, synchronized in G₀ or in S phase were immunoprecipitated with an RNA polymerase II specific antibody, an antiacetyl-histone H3 or a β-actin antibody as a negative control. Precipitated DNA or 10% of chromatin input was amplified with gene specific primers for *skp2* or for *cyclin A*. (d) Chromatin of MiaPaCa2 cells, synchronized in G₀ or S phase immunoprecipitated with an HDAC1, HDAC3 or a β-actin antibody as a negative control. Precipitated DNA or 10% of the chromatin input was amplified with gene specific primers for *skp2* or *GAPDH*.

a 3-fold increase of *skp2* mRNA in early S phase compared to G₀ phase. Furthermore, binding of RNA-polymerase II to the proximal *skp2* gene promoter was increased and correspondingly an increase of acetylated histone H3 at the proximal *skp2* gene promoter was observed (Fig. 2c). As a control, identical changes were found at the *cyclin A* gene. Because HDAC1 and HDAC3 are known to regulate cell cycle progression, binding to the proximal *skp2* gene promoter was

tested. We found binding of HDAC1 in the proximal position in the G₀ phase, disappearing in early S phase (Fig. 2d). Inversely, HDAC3 binding to the proximal *skp2* gene promoter increased in early S phase, suggesting an activating function of HDAC3 for *skp2* gene transcription (Fig. 2d). HDAC1 and HDAC3 binding was not observed at the GAPDH promoter, demonstrating specificity. Protein abundance of HDAC1 and HDAC3 was not significantly altered in G₀ and S phases (Fig. 2a).

Knockdown of HDAC3 impairs *skp2* gene transcription

To investigate the role of HDAC3 of *skp2* gene transcription in more detail, we used RNA interference techniques. Forty-eight hours after transfection of the MiaPaCa2 cells with HDAC1 and HDAC3 siRNAs, a knockdown was observed compared to control siRNA transfected cells (Fig. 3a). To test the effect(s) of this knockdown on cell proliferation, BrdU incorporation assays were performed. Whereas HDAC3 knockdown reduced BrdU incorporation to 57% compared to untreated controls, BrdU incorporation was only marginally affected by HDAC1 knockdown (Fig. 3b). Skp2 protein expression was down-regulated in HDAC3 siRNA transfected, but not in HDAC1 siRNA transfected cells (Fig. 3a). Fitting to reduced *skp2* expression, p27^{Kip1} protein abundance was found to be increased in HDAC3 siRNA transfected cells (Fig. 3a). In addition to *skp2*, transfection of the HDAC3 siRNA also resulted in a decrease of HDAC1 protein expression (Fig. 3a). Furthermore, *skp2* mRNA expression was reduced 48 h after transfection of the HDAC3 specific siRNA compared to untransfected controls (Fig. 3c). No significant down-regulation of *skp2* mRNA expression was observed after transfection of a control or HDAC1 siRNA (Fig. 3b). In line with reduced *skp2* protein and mRNA abundance, decrease in binding of RNA polymerase II to the proximal *skp2* gene promoter after transfection of HDAC3 siRNA was observed (Fig. 3d). No change of RNA polymerase II binding to proximal *skp2* gene promoter was detected after transfection of HDAC1 siRNA. RNA polymerase II binding to the GAPDH promoter was unchanged by HDAC1 and HDAC3 siRNA transfection, demonstrating specificity. Furthermore, cotransfection of the *skp2*-273/+244Luc reporter gene construct with a HDAC3 expression vector leads to increased *skp2* promoter activity in a dose-dependent fashion (Fig. 3e). Skp2 promoter activity increased approximately 2-fold with the highest quantity of HDAC3 expression plasmid used. In contrast, the pGL3control reporter was down-regulated by the cotransfection of HDAC3, demonstrating specificity (Fig. 3e).

DISCUSSION

Although p27^{Kip1} participates in HDAC inhibitor-induced cell population growth retardation, the molecular mechanisms had not been investigated in detail. Our data now provide evidence that TsA down-regulates *skp2* transcription and thereby up-regulates p27^{Kip1} protein levels indirectly, by a post-translational mechanism. Furthermore, we show that HDAC3 maintains *skp2* transcription.

Recently, Chen & Faller have reported that HDAC inhibition induces post-translational elevation of p27^{Kip1} and that p27^{Kip1} is essential for HDAC inhibitor-induced G₁-phase arrest, because p27^{Kip1} deficient fibroblasts were resistant to HDAC inhibition-induced arrest (Chen & Faller 2005). This observation is in line with our data, demonstrating elevation of p27^{Kip1} after TsA treatment, without change of p27^{Kip1} mRNA levels. Phosphorylated p27^{Kip1} is specifically recognized by F-box protein *skp2*, a receptor component of SCF E3-ubiquitin ligase, and is subsequently degraded in the ubiquitin-proteasome pathway (Nakayama & Nakayama 2005).

Figure 3. Knockdown of HDAC3 impairs *skp2* gene transcription. (a) Western blots of HDAC1, HDAC3, skp2 and p27^{Kip1} expression 48 h after transfection of subconfluent MiaPaCa2 cells with a HDAC1-specific siRNA (lane 2), a HDAC3-specific siRNA (lane 3) or a control siRNA (lane 1). The right graph shows quantification of skp2 expression of four independent experiments (Student's *t*-test: **P* = 0.002 versus controls). (b) BrdU incorporation in MiaPaCa2 cells 48 h post-transfection of subconfluent cells with indicated siRNAs (Student's *t*-test: **P* < 0.001 versus controls). (c) mRNA abundance of *skp2* was quantified using real-time PCR analysis and normalized to cyclophilin expression levels 48 h after the transfection of subconfluent MiaPaCa2 cells as indicated (Student's *t*-test: **P* < 0.001 versus controls). (d) Subconfluent MiaPaCa2 cells transfected with the indicated siRNAs. After 48 h, chromatin was immuno-precipitated with an RNA polymerase II specific antibody or a β-actin antibody as a negative control. Precipitated DNA or 10% of the chromatin input was amplified with gene specific primers for *skp2* or *GAPDH* as control. (e) 500 ng of the pGL3 control or the *skp2*-273/+244Luc vector were cotransfected with increasing amounts of an HDAC3 expression plasmid into MiaPaCa2 cells, as indicated. Amount of DNA was kept constant using pcDNA3. Twenty-four hours after the transfection, luciferase activity was measured (Student's *t*-test: **P* < 0.001 versus controls; ***P* = 0.003 versus controls).

Down-regulation of *skp2* protein and mRNA abundance after TsA treatment in our model indicates that HDAC inhibitor-induced transcriptional down-regulation of *skp2* is responsible for stabilization of p27^{Kip1}.

Effects of HDAC inhibitors concerning *skp2* expression are conflicting. Reduced expression of *skp2* mRNA and protein abundance after treatment of HeLa cells with sodium butyrate has been reported (Finzer *et al.* 2001). In contrast, TsA treatment resulted in an increase of *skp2* expression in MCF7 cells (Alao *et al.* 2004). We now clearly demonstrate that HDACs control transcription of *skp2* in our cellular model. HDAC1 was mainly associated with the *skp2* gene in the G₀ phase of the cell cycle, implicating a suppressive function, and interestingly, *skp2* was found to be an E2F target (Zhang & Wang 2006). Because E2F transcription factors are controlled by pocket proteins like Rb, and Rb is known to recruit HDAC1 into repressive complexes, a trimolecular complex including E2F, Rb and HDAC1 is most likely to repress *skp2* in G₀ phase. Although the observation of HDAC1 binding to *skp2* gene in the G₀ phase suggests suppressive function, our data are correlative and the pathway suggested awaits further experimental validation. In contrast to reports demonstrating impaired proliferation due to up-regulation of p21^{WAF1} and p27^{Kip1}, we have found no influence of HDAC1 for regulation of cell proliferation in our model (Lagger *et al.* 2002). Because loss of HDAC1 is compensated for by increased HDAC2 expression and HDAC2 can regulate HDAC1 target genes in the absence of HDAC1, acquisition of HDAC1s proliferative function by HDAC2 can explain unaltered cell proliferation after the HDAC1 knockdown in our system (Lagger *et al.* 2002; Zupkovitz *et al.* 2006). Furthermore, cell-type and tissue-specific mechanisms might contribute to the influence of HDAC1 in cell cycle control.

Different from HDAC1, HDAC3 was discovered to mainly be bound to the *skp2* promoter in S phase, arguing for an activating function of HDAC3 for *skp2* transcription. This is further supported by reduced *skp2* mRNA and protein levels as well as by impaired binding of RNA polymerase II to the *skp2* promoter, detected after knockdown of HDAC3. Furthermore, the proximal *skp2* promoter was directly activated by HDAC3. Dependency of gene transcription on HDAC3 is an unexpected finding; however, there are numerous examples where HDACs are required for gene activation (Lallemant *et al.* 1996; Laribee & Klemsz 2001; Wang *et al.* 2002; Ferguson *et al.* 2003; Xu *et al.* 2003; Qiu *et al.* 2006). Molecular mechanisms of HDAC-dependent transcription are not entirely clear, but seem to involve deacetylation of non-histone proteins. Thus, IL-3-dependent transcription of *Id-1* gene requires HDAC1 mediated deacetylation of the C/EBP β transcription factor, whose DNA-binding affinity is diminished by acetylation (Xu *et al.* 2003). The HDAC3 target, mediating *skp2* promoter activation in our model system is unknown in the moment. In addition to HDAC3 binding to the *skp2* promoter in S phase of the cell cycle, we found increased acetylation of histone H3. This suggests that HDAC3 binding to the *skp2* promoter does not exclude histone acetylation and gene activation. In line, INF γ stimulates acetylation of histone H4 and binding of HDAC1 to the *Irf1* gene promoter leading to *Irf1* gene expression (Zupkovitz *et al.* 2006). After transfection of HDAC3 specific siRNA into MiaPaCa2 cells, we also detected reduced protein expression of HDAC1. As discussed, our data suggest suppressive function of HDAC1 for the *skp2* gene. Selecting for low HDAC1 expression in HDAC3 siRNA transfected MiaPaCa2 cells might be a compensatory mechanism to maintain *skp2* gene expression and proliferation. Alternatively, the *HDAC1* gene could be a *HDAC3* target gene. These explanations and suggestions await further experimental validation.

In summary, our work connects HDAC3 to transcription of the oncogene *skp2*. Because *HDAC3* is one of the commonly overexpressed genes in tumours, activation of an HDAC3-*skp2* pathway might be a frequent feature of human cancers (Pilarsky *et al.* 2004).

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