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Abstract

SRSF2 is a Ser-Rich/Arg protein belonging to the family of SR proteins that are crucial regulators of constitutive and alternative pre-mRNA splicing. Although it is well-known that phosphorylation inside RS domain controls SR proteins activity, other post-translational modifications regulating SRSF2 functions have not been described to date. In this study, we provide the first evidence that the acetyltransferase Tip60 acetylates SRSF2 on its lysine 52 residue inside the RNA Recognition Motif, and promotes its proteasomal degradation. We also demonstrate that the deacetylase HDAC6 counters this acetylation and acts as a positive regulator of SRSF2 protein level. In addition, we show that Tip60 down-regulates SRSF2 phosphorylation by inhibiting the nuclear translocation of both SRPK1 and SRPK2 kinases. Finally, we demonstrate that this acetylation/phosphorylation signaling network is required for SRSF2 accumulation and for the control of *caspase-8* pre-mRNA splicing in response to cisplatin, and directs by this way the cell fate, growth arrest versus apoptosis. Taken together, these results unravel lysine acetylation as a crucial post-translational modification regulating SRSF2 protein activity in response to genotoxic stress.

Key Words: Acetylation/Phosphorylation/SRSF2/SRPK/Tip60.

Category: Proteins/Differentiation & Death

Introduction

Pre-mRNA splicing consists of a highly regulated cascade of events that are critical for gene expression in higher eukaryotic cells. This process has emerged as an important mechanism of genetic diversity as about 74% of human genes undergo alternative splicing, leading to the synthesis of various protein isoforms with different biological properties (Johnson *et al*, 2003). SRSF2 belongs to the serine/arginine-rich (SR) protein family, one of the most important class of splicing regulators that play a prominent role in splice-site selection, in multiples steps of spliceosome assembly as well as in both constitutive and alternative splicing (Graveley, 2000). All members of the SR protein family share a modular organization and contain one or two N-terminal RNA recognition motifs (RRM) that interact with the pre-mRNA and influence substrate specificity (Liu *et al*, 1998), as well as a C-terminal serine/arginine-rich sequence known as the RS domain that functions as a protein-protein interaction module (Wu and Maniatis, 1993). SR proteins activity is highly regulated by extensive and reversible phosphorylation of serine residues inside RS domain. These phosphorylations modulate protein-protein interactions within the spliceosome and can influence RNA-binding specificity, splicing activity and sub-cellular localization. To date, numerous kinases phosphorylating SR proteins have been identified. They include the SRPK and CLK/STY family kinases that are highly specific for RS domain–containing splicing factors, the DNA topoisomerase I and AKT (Stamm, 2008). However, data addressing the cellular signals that control phosphorylation of SR proteins remain scarce, as well as the specific kinases involved in these effects.

Chromatin biology and pre-mRNA splicing have been considered for a long time as two independent fields. However, recently, chromatin structure has been shown to affect both constitutive and alternative splicing, either through the recruitment of splicing factors (Sims *et*

al, 2007) or through the modulation of RNA polymerase II elongation rate (Batsche *et al*, 2006). In addition, two studies have demonstrated that DNA sequences associated with nucleosomes are preferentially located in exons (Schwartz *et al*, 2009; Tilgner *et al*, 2009), providing a general concept for how the architecture of genome packaging could influence pre-mRNA splicing. Chromatin structure is highly controlled by post-translational modifications of histone proteins tails including phosphorylation or acetylation. These modifications are catalyzed by chromatin-modifying enzymes that add or remove specific groups in a reversible way. It was recently reported that two SR proteins, namely SRSF3 and SRSF1, bind histone H3 tail to control cell cycle progression (Loomis *et al*, 2009). These data provide the first evidence that SR proteins associate with chromatin, and suggest that they could also be directly targeted by components of chromatin-remodeling complexes. Lysine acetylation is highly regulated through the opposite actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs) enzymes. Besides histones, an increasing number of cellular proteins are also subjected to lysine acetylation (Yang and Seto, 2008). Recently, a high-resolution mass spectrometry analysis revealed that a large number of acetylation sites are present on proteins implicated in splicing, including SR proteins, and identified the RNA recognition motif as a major domain for acetylation (Choudhary *et al*, 2009). These data support the idea that lysine acetylation could regulate SR proteins function. In this study, we demonstrate for the first time that an acetylation/phosphorylation network controls the turn-over and activity of the splicing factor SRSF2 in response to genotoxic stress. Therefore, besides phosphorylation, lysine acetylation also appears as a crucial post-translational modification of SR proteins.

Results

SRSF2 but not SRSF1 is acetylated by the histone acetyltransferase Tip60

To test whether SRSF2 could be acetylated, we first transfected SAOS2 cells with an expression vector encoding HA-tagged SRSF2 protein and used anti-HA antibody to immunoprecipitate SRSF2 which acetylated status was analyzed by western blotting using an anti-acetyl-lysine antibody. As shown in Figure 1A, an acetylated form of SRSF2 was detected in these conditions. To go further, immunoprecipitation with anti-acetyl-lysine (Figure 1B, left panel) or anti-SRSF2 (Figure 1B, right panel) antibody was performed in nuclear-enriched extracts from H69 cells that express high levels of SRSF2. Again, an acetylated form of SRSF2 was detected by western blotting using an anti-SRSF2 or an anti-acetyl-lysine antibody, respectively. Together, these data provide the first evidence that SRSF2 is subjected to acetylation. During the course of this study, we demonstrated that the Tip60 acetyltransferase controls acetylation of non histone proteins in our cellular models (Leduc *et al*, 2006; Van den Broeck *et al*, in prep) and we postulated that SRSF2 could be a new Tip60 target. To test this hypothesis, an *"in vitro"* acetyltransferase assay was set-up in which various truncated GST-SRSF2 fusion proteins were used as putative substrates for the Tip60 acetyltransferase (His-Tip60$^{212-513}$) in the presence of C^{14}-Acetyl-CoA (Figure 1C). Of note, truncated fragments of SRSF2 were used in this assay as full-length recombinant SRSF2 protein cannot be produced in bacteria. As detected by autoradiography (Figure 1D, upper panel), recombinant Tip60 was able to efficiently acetylate SRSF2 on its first sixty amino acids (1-60) encompassing the RNA Recognition Motif (RRM). This effect was specific of Tip60 since another acetyltransferase, namely GCN5, was unable to acetylate the GST-SRSF2(1-60) fragment (Figure 1D, upper panel). No signal was detected with the GST-SRSF2(60-115) truncated fragment while a very faint signal, as compared to GST-SRSF2(1-60) fragment, was detected with the GST-

SRSF2(115-221) truncated protein (Figure 1D, upper panel), suggesting that SRSF2 could also be acetylated by Tip60 inside its RS domain part. By contrast, when the recombinant SRSF1 protein was used as an *in vitro* substrate of either Tip60 or GCN5 acetyltransferase, acetylation could not be detected (Supplementary Figure 1). Although these results did not exclude the possibility that SRSF1 could also be subjected to acetylation (Choudhary *et al*., 2009), they indicate that SRSF1 is not a substrate of Tip60 *in vitro*. In order to confirm these results *"in vivo"*, Tip60 was knocked-down in H1299 cells and the acetylated status of endogenous SRSF2 was studied by immunoprecipitation using either an anti-acetyl-lysine (Figure 1E, upper panel) or an anti-SRSF2 (Figure 1E, lower panel) antibody followed by immunoblotting using an anti-SRSF2 or an anti-acetyl-lysine antibody, respectively. The data demonstrated that Tip60 neutralization significantly decreases SRSF2 acetylation in these cells. By contrast, and consistent with the *in vitro* experiments, acetylation of SRSF1 could not be detected when the membrane loaded with the anti-acetyl-lysine immunoprecipitates was re-blotted with an anti-SRSF1 antibody (Figure 1E, upper panel). In addition, an interaction between both Tip60 and SRSF2 proteins was depicted in cells co-transfected with SRSF2- and Tip60-encoding plasmids (Supplementary Figure 2), indicating that both proteins exist in a complex within these cells. Overall, these results demonstrate that SRSF2 is a substrate of the acetyltransferase Tip60. SRSF2(1-60) N-terminal part contains three lysine (K) residues (K17, K36 and K52) (Figure 1F, upper panel). To map the acetylation site(s) targeted by Tip60 on SRSF2 inside its RRM domain, we produced recombinant mutated GST-SRSF2(1-60) fusion proteins in which each lysine was substituted by an arginine (R), a non-acetylable residue. Analysis of the K52R and K17R point mutants indicated that K52 was a prime-candidate site for Tip60-mediated acetylation since autoradiographic signal was dramatically lost when K52 residue was mutated into arginine (Figure 1F, lower panel). Of note, we were not able to produce the GST-SRSF2(1-60) K36 point mutant in bacterial system, so we cannot exclude the possibility that

K36 could be acetylated. Taken together, these results demonstrate that Tip60 acetylates SRSF2 on its lysine K52 residue.

Acetylation by Tip60 controls SRSF2 protein turn-over

In order to investigate the biological consequences of Tip60-mediated SRSF2 acetylation, we knocked-down Tip60 expression by using two distinct siRNA in various carcinoma cell lines and monitored the expression level of SRSF2. Immunoblotting experiments reproducibly demonstrated that SRSF2 protein level significantly increased when Tip60 was neutralized as compared to control cells (Figure 2A and Supplementary Figure 3A). This effect was specific of SRSF2 since SRSF1 protein amount was apparently not affected. This effect also mainly occurred at a post-transcriptional level since *SRSF2* mRNA did not reproducibly increase under these conditions (Figure 2A and Supplementary Figure 3B). In order to confirm these results, rescue experiments were performed in which either the wild-type Tip60 or a mutant Tip60 with a defective HAT activity ($Tip60^{G380}$) was overexpressed in Tip60 knock-out cells. As shown in Figure 2B, restoration of Tip60 expression in Tip60-deficient cells prevented SRSF2 protein accumulation while the mutant $Tip60^{G380}$ did not. Moreover and consistent with Tip60 being a negative regulator of SRSF2, SRSF2 protein level decreased when cells were co-tranfected with Tip60 encoding plasmid (Figure 2C). Interestingly, treating cells with the proteasomal inhibitor MG132 prevented SRSF2 downregulation in that case, thereby indicating that SRSF2 could be subjected to a Tip60-mediated proteasomal degradation. By contrast, cotransfection with the mutant $Tip60^{G380}$ encoding plasmid had no significant effect on exogenous SRSF2 protein level (Figure 2C). Similar results were obtained when the effect of Tip60 or $Tip60^{G380}$ was assessed on endogenous SRSF2 protein level (Figure 2D). Taken together, these data support the notion that Tip60-mediated SRSF2 acetylation controls SRSF2 turn-over. To assess the role of lysine K52 in this setting, we generated an expression vector encoding a HA-tagged

SRSF2(K52R) mutant protein harbouring a K52R substitution, thereby mimicking a non Tip60-acetylable form of SRSF2. In co-transfection experiments, Tip60 as well as Tip60^{G380} were unable to modify the level of the SRSF2(K52R) mutant (Figure 2E). To confirm the role of the K52 residue on SRSF2 half-life, H1299 cells were transfected with plasmid encoding either HA-tagged wild-type (WT) or mutant (K52R) SRSF2 protein and incubated with cycloheximide (CHX), a widely used inhibitor of translation. The amount of HA-SRSF2 WT or HA-SRSF2 (K52R) protein was then analyzed by immunoblotting. As depicted in Figure 2F, the mutant SRSF2(K52R) half-life was strongly enhanced as compared to that of wild-type SRSF2. When similar experiments were performed in HA-Tip60 transfected cells, Tip60 was found to reduce the half-life of HA-SRSF2 protein, while it did not affect that of the HA-SRSF2(K52R) mutant (Supplementary Figure 4). Overall, these results demonstrate that acetylation of SRSF2 by Tip60 negatively controls the expression level of the SRSF2 protein by a mechanism involving the proteasome pathway.

The deacetylase HDAC6 positively controls SRSF2 protein level

Lysine acetylation is finely regulated by the equilibrium between acetyltransferase and deacetylase activities. Among histone deacetylases, HDAC6 is unique as it possesses intrinsic ubiquitin-linked functions in addition to its deacetylase activity (Seigneurin-Berny *et al*, 2001; Boyault *et al*, 2006), and has been reported to protect ubiquitinated proteins from subsequent processing by the proteasome (Boyault *et al*, 2006; Boyault *et al*, 2007a). Having demonstrated that acetylation controls SRSF2 protein turn-over via a proteasome-dependent mechanism, we investigated the role of HDAC6. First, HA-tagged SRSF2 was expressed in the presence or absence of HDAC6. As shown in Figure 3A, overexpressing HDAC6 induced a strong accumulation of SRSF2 protein without affecting its mRNA level (Supplementary Figure 5A). Conversely, neutralizing HDAC6 by using siRNA significantly decreased SRSF2 protein

expression, but not *SRSF2* mRNA or SRSF1 protein level (Figure 3B and data not shown). Overall, these data indicated that HDAC6 positively controls SRSF2 expression level. In addition, both HDAC6 and SRSF2 proteins were found to co-immunoprecipitate (Supplementary Figure 5B). In order to investigate the role of lysine K52 in this setting, we generated an anti-Ac-K52 SRSF2 antibody recognizing SRSF2 acetylated onto K52 (Supplementary Figure 6). Immunoprecipitation experiments of acetylated K52-SRSF2 were carried-out in cells deprived of HDAC6 followed by immunoblotting directed against SRSF2. The results showed that knocking-down HDAC6 induced the accumulation of an acetylated form of SRSF2 (Figure 3C, upper panel). Interestingly, HDAC6 was unable to stabilize the mutant SRSF2(K52R) as compared to the wild-type SRSF2 protein (Figure 3C, middle panel). In addition, the knock-down of HDAC6 in cells overexpressing either the SRSF2 or mutant SRSF2(K52R) construct severely decreased wild-type SRSF2 protein level while it had no effect on SRFS2(K52R) protein expression (Figure 3C, lower panel). Taken together, these results indicate that HDAC6 targets lysine K52 to positively control SRSF2 protein expression. To extend these data, we took advantage of 3T3 cell lines previously established from mouse embryo fibroblasts isolated from parental (WT) or HDAC6-deficient (KO) mice in which wild-type HDAC6 was re-expressed (+WT) (Boyault *et al*, 2007a), and analyzed expression of murine SRSF2 by immunoblotting. As expected from the above data, SRSF2 protein level was significantly decreased in HDAC6-/- 3T3 as compared to parental cells, and reintroduction of wild-type HDAC6 in knock-out cells restored the basal level of SRSF2 protein (Figure 3D, upper panel). HDAC6 is a unique class II deacetylase which contains two catalytic domains, and a C-terminal zinc finger domain (ZnF-UBP) that binds with high-affinity free ubiquitin as well as mono- and polyubiquitinated proteins (Boyault *et al*, 2007b). In order to characterize the functional domains of HDAC6 involved in SRSF2 regulation, we monitored the expression of SRSF2 in 3T3 cells derived from HDAC6-deficient mice re-expressing either the wild-type

HDAC6 (+WT), the HDAC6 mutant deprived of deacetylase activity (+HD^{m}), or the HDAC6 mutant deprived of ubiquitin binding function (+Ub^{m}). The results showed that both mutants were unable to rescue the down-regulation of SRSF2 upon HDAC6 loss as compared to wild-type HDAC6 (Figure 3D, lower panel). In addition, HDAC6 was absolutely required for the accumulation of SRSF2 in 3T3 cells treated with MG132 (Figure 3E). This effect was dependent on both the catalytic activity and the ability of HDAC6 to bind ubiquitin. Overall, these results demonstrate that HDAC6 is a key actor of SRSF2 protein turn-over and identify the crucial role of its catalytic and ubiquitin-binding domains in this setting. Finally, as the ratio between Tip60 and HDAC6 proteins could be a determinant of SRSF2 expression, we performed co-transfection experiments in which increasing amounts of HDAC6 were produced in the presence of high levels of Tip60. As shown in Figure 3F (upper panel), HDAC6 thoroughly reversed Tip60-mediated down-regulation of SRSF2, even at a low level, indicating that HDAC6 activity outweighs the negative regulation of SRSF2 imposed by Tip60. By contrast, and consistent with K52 being a key residue in this setting, HDAC6 did not affect the expression level of SRSF2(K52R) in cells overexpressing Tip60 (Figure 3F, lower panel). As a whole, our data support a model in which HDAC6 plays a key role in the maintenance of SRSF2 protein level by inhibiting Tip60-mediated acetylation and proteasomal degradation. These results are the first evidence that an acetylation/deacetylation switch controls SRSF2 protein turn-over.

Tip60 inhibits SRSF2 phosphorylation by preventing SRPK1 and SRPK2 nuclear translocation

Numerous studies have now identified cross-talks between lysine acetylation and others post-translational modifications, such as phosphorylation (Yang and Seto, 2008). As phosphorylation is a critical regulator of SR proteins activity, we then asked whether

acetylation could affect the phosphorylation status of SRSF2. To explore this possibility, we used two different antibodies recognizing phosphorylated forms of SRSF2 (Figure 4A and Supplementary Figure 7) and we first analyzed the phosphorylation level of SRSF2 in cells transfected with a plasmid encoding Tip60-HA, in the presence of MG132 to prevent SRSF2 proteasomal degradation. Overexpression of Tip60 significantly attenuated SRSF2 phosphorylation (Figure 4A). The effect of Tip60 required its HAT activity since SRSF2 phosphorylation was not affected by mutant Tip60^{G380}. In order to confirm these results, HA-tagged SRSF2 protein was overexpressed together with or without Tip60 in cells treated with MG132, and its phosphorylated status was assessed after immunoprecipitation with an anti-HA antibody followed by immunoblotting using the anti-phospho SRSF2 antibody. As shown in Figure 4B, Tip60 overexpression strongly decreased the phosphorylation of SRSF2. In addition, phospho-SRSF2 protein level was enhanced when Tip60 was neutralized by siRNA (Figure 4C). Altogether, these data indicate that Tip60 negatively controls SRSF2 phosphorylation and suggest a dialogue between acetylation and phosphorylation networks to control SRSF2 protein activity. Among the growing list of SR protein kinases, the SRPK family that includes SRPK1 and SRPK2 is well-characterized. Interestingly, we provided evidence that down-regulation of either SRPK1 or SRPK2 protein attenuated SRSF2 phosphorylation, whereas treatment with TG003, a pharmacological inhibitor of CLK/STY, another SR-phosphorylating kinase, had no effect (Supplementary Figure 8). Overall, these data indicate that both SRPK1 and SRPK2 kinases largely contribute to SRSF2 phosphorylation in our cellular models. Therefore, to test whether Tip60 could prevent SRSF2 phosphorylation by altering the expression level of SRPK(s), we performed immunoblotting experiments using whole-cell extracts of cells in which Tip60 expression was either upregulated or knocked-down. As shown in Figures 4D and 4E (upper panels), overexpression or neutralization of Tip60 did not significantly modify the total amount of SRPK1 or SRPK2

protein. SRPK1 and SRPK2 kinases are proteins that shuttle between the cytoplasm and the nucleus (Ding *et al*, 2006). So, we next tested whether Tip60 could alter SRPK(s) sub-cellular distribution. To this purpose, cellular fractionation experiments were set-up in cells transfected with either Tip60 encoding plasmid or *Tip60* siRNA, and SRPK protein levels were analyzed by immunoblotting in nuclear or cytoplasmic fractions. A significant decrease of nuclear SRPK(s) correlated with a decreased level of nuclear P-SRSF2 protein was observed upon Tip60 overexpression as compared to control cells (Figure 4D, lower panel). By contrast, strong nuclear accumulation of SRPK(s) associated with P-SRSF2 increase was observed in cells knocked-down for Tip60 as compared to *mismatch* transfected cells (Figure 4E, lower panel). In order to confirm these results, immunofluorescence studies were set-up. As shown in Figure 4F, the nuclear amount of both SRPK1 and SRPK2 proteins increased in cells deprived of Tip60 as compared to *mismatch* transfected cells. Conversely, a slight but reproducible decrease of both SRPK1 and SRPK2 nuclear staining was observed in cells overexpressing Tip60, whereas this effect was not observed with the mutant $Tip60^{G380}$ (Supplementary Figure 9). Altogether, these results indicate that Tip60 negatively controls SRSF2 phosphorylation and the nuclear accumulation of SRPK kinases, by a mechanism requiring its HAT activity. Of note, HDAC6 neither affected SRSF2 phosphorylation status, nor modified SRPK(s) protein levels or sub-cellular localization in our cellular models (data not shown).

SRSF2 accumulates in a hypoacetylated/phosphorylated form in response to cisplatin treatment

We finally attempted to characterize in which physiological context the signaling networks involving Tip60, HDAC6 and SRPK(s) proteins controlled SRSF2 protein status. We recently demonstrated that SRSF2 is upregulated and required for the apoptotic response induced by alkylating agents (Merdzhanova *et al*, 2008). In this study, we provided evidence that SRSF2

also accumulates following cisplatin treatment (Figure 5A, left panel). As RT-QPCR experiments failed to detect an increase of *SRSF2* mRNA level in treated cells (Supplementary Figure 10A), the accumulation of SRSF2 protein in response to cisplatin is mainly a post-transcriptional event. In addition, inhibition of SRSF2 expression strongly impaired the occurrence of apoptosis induced by cisplatin (Figure 5A). The use of a distant splice donor site at the 3'-end of exon 8 of human *caspase-8* pre-mRNA leads to the synthesis of an alternative splice variant, *caspase-8L*, a competitive inhibitor of caspase-8 (Himeji *et al*, 2002). In our cells, cisplatin decreased the *caspase-8L*/*caspase-8a* ratio (Figure 5B). This effect was prevented when SRSF2 was neutralized, indicating that SRSF2 is involved in *caspase-8* pre-mRNA splicing upon cisplatin treatment. Of note, caspase-8 was required for apoptosis in this setting, since co-treating cells with cisplatin and the specific cell-permeable caspase-8 inhibitor IETD-CHO significantly decreased the number of apoptotic cells and prevented the decrease of the *caspase-8L*/*caspase-8a* ratio (Supplementary Figures 10C and 10D). Taken together, these results indicate that SRSF2 accumulates upon cisplatin treatment and contributes to cisplatin-induced apoptosis. In order to assess whether acetylation/phosphorylation signaling networks could play a role in this context, we first analyzed the acetylated status of SRSF2. Immunoprecipitation experiments demonstrated that SRSF2 acetylation was dramatically lost upon cisplatin treatment (Figure 5C). This effect was specific of SRSF2 since cisplatin strongly stimulated the acetylation of the transcription factor E2F1, in agreement with previous data (Ianari *et al*, 2004). Consistent with the loss of SRSF2 acetylation, immunoblotting and RT-QPCR experiments demonstrated that cisplatin induced a significant decrease of both Tip60 mRNA and protein levels as compared to untreated cells, which correlated with the occurrence of apoptosis as detected by caspase-3 activation (Figure 5D). These results were also confirmed in H358 cells (Supplementary Figure 10B). It was recently shown that the circadian transcription factor Clock controls *Tip60* gene expression in human lung carcinoma cell lines, and that high levels of both Clock and Tip60 proteins correlate with resistance to cisplatin

(Miyamoto *et al*., 2008). Interestingly, we observed that down-regulation of *Tip60* mRNA and protein upon cisplatin-induced apoptosis was associated with the decrease of Clock protein level (Figure 5D). As cisplatin did not affect Tip60 mRNA stability as measured after actinomycin D treatment (Supplementary Figure 11), our results suggest that Clock might be involved in the decrease of *Tip60* gene expression following cisplatin exposure. Moreover, while expression of HDCA6 protein did not significantly vary in response to cisplatin, its neutralization strongly prevented the accumulation of SRSF2 (Figure 5E, upper panel), indicating that HDAC6 is involved in SRSF2 increase in that case. These results were confirmed in 3T3 cells (Figure 5E, lower panel). Finally, and concomitant with the loss of SRSF2 acetylation, increased levels of phosphorylated SRSF2 protein (P-SRSF2) were detected in response to cisplatin (Figure 5F) and were associated with the nuclear accumulation of both SRPK1 and SRPK2 kinases, as detected using both sub-cellular fractionation experiments (Figure 5G) and immunofluorescence studies (Supplementary Figure 12). Overall, these results demonstrate that cisplatin induces the accumulation of a hypoacetylated and phosphorylated form of SRSF2, by a mechanism involving the Tip60, HDAC6, SRPK1 and SRPK2 proteins.

SRPK1 and SRPK2 differentially control SRSF2 phosphorylation and cell fate in response to cisplatin

It has been proposed that SRPK1 and SRPK2 kinases have both overlapping and unique functions in mammalian cells (Mathew *et al*, 2008). Finally, we investigated the role of each SRPK in cisplatin-induced SRSF2 phosphorylation and apoptosis. To this purpose, H358 (Figure 6) and H1299 (data not shown) cells were transfected with siRNA targeting either *SRPK1* or *SRPK2* and were subjected or not to cisplatin exposure for 24 hours. We observed that neutralization of SRPK2 significantly prevented the phosphorylation of SRSF2 (Figure

6A, left panel) and the induction of apoptosis following cisplatin treatment (Figure 6A, left and right panels). Of note, and as shown by western blotting (Figure 6A, left panel), exposure to cisplatin led to the appearance of a cleaved product of SRPK2 that has been previously described in apoptotic cells (Kamachi *et al*, 2002). In addition, and consistent with apoptosis measurements, the knockdown of SRPK2 strongly increased the *caspase-8L*/*caspase-8a* ratio in cisplatin-treated cells (Figure 6B). Similar results were obtained in H1299 cells (data not shown). Altogether, these results demonstrate that SRPK2 is a critical regulator of SRSF2 during cisplatin-induced apoptosis. By contrast, the neutralization of SRPK1 did not prevent the accumulation of P-SRSF2, and apoptosis sharply increased as detected by western blot (Figure 6A, left panel) or flow cytometry (Figure 6A, right panel). These data indicate that SRPK1 inhibits rather than stimulates cisplatin-induced apoptosis. Therefore it appears that SRPK1 and SRPK2 kinases do not play the same role in response to cisplatin. Finally, as apoptosis was impaired in SRPK2-depleted cells upon drug treatment, we wondered about the fate of these cells. To answer, the cell cycle distribution was analyzed by flow cytometry. As depicted in Figure 6C, while appearance of apoptotic cells with a sub-G_1 DNA content was observed after cisplatin exposure, inhibiting SRPK2 expression in these conditions induced a G_2/M arrest. Overall, these data indicate that SRPK-regulated SRSF2 phosphorylation controls cell fate decision in response to cisplatin treatment.

Discussion

A high-resolution mass spectrometry analysis recently identified 3600 lysine acetylation sites on 1750 proteins (Choudhary *et al*, 2009). Interestingly, when these proteins were classified according to their cellular functions, two classes emerged: the first one bringing together cell cycle regulators and the second one, RNA splicing components. Moreover, when architecture

of acetylated proteins was analyzed, RNA recognition motif (RRM) domains were overrepresented in the lysine acetylome, strongly supporting a role of this post-translational modification in the control of pre-mRNA processing events. In this study, we unravel the first acetylation/phosphorylation signaling network regulating the turn-over and activity of a key splicing regulator, namely the SR protein SRSF2. In this setting, we identify a closed interplay between SRSF2, the Tip60 acetyltransferase, the HDAC6 deacetylase and the SRPK1 and SRPK2 kinases. Therefore, besides phosphorylation, we characterize lysine acetylation as a new post-translational modification of SR proteins, which role as a regulator of pre-mRNA splicing events has now to be fully considered.

Only a few data exist about the molecular mechanisms regulating SRSF2 protein expression. It is known that, when highly expressed, SRSF2 negatively controls its own expression, through activation of alternative splicing events leading to the generation of distinct splice variants that are targeted by the non-sense-mediated mRNA decay machinery (Sureau *et al*, 2001). In addition, we recently showed that E2F1 is a direct transcriptional factor towards *SRSF2* promoter (Merdzhanova *et al*, 2008), thereby identifying an upstream activator of SRSF2. In this study, we demonstrate that Tip60 acetylates SRSF2 on its lysine 52 (K52) residue and promotes its proteasomal degradation, thereby characterizing a novel mechanism that controls SRSF2 protein level. It has been previously described that SRSF2 partially colocalizes with 20S proteasomes and accumulates under treatment with the proteasome inhibitor lactacystin (Dino Rockel and von Mikecz, 2002). Moreover, another SR protein, namely SRSF6, has been reported to be targeted by the proteasome (Lai *et al*, 2003). Therefore, these and our data suggest that proteasome-dependent proteolysis could be a general mechanism to control SR proteins turn-over. Most of the examples described in the literature designate lysine acetylation as a stabilizing post-translational modification, through a blocking effect which hinders lysine

ubiquitination (Sadoul *et al*, 2008). In contrast and consistent with our results, lysine acetylation has also been reported as a signal enhancing protein degradation (Caron *et al*, 2005; Leduc *et al*, 2006; Sadoul *et al*, 2008). We do not elucidate here the molecular mechanisms by which Tip60 targets SRSF2 to proteasomal degradation, but it has been previously shown that Tip60 can interact with some E3-ubiquitin ligases such as MDM2 (Legube *et al*, 2002) or Pirh2 (Logan *et al*, 2004). It is thus tempting to speculate that Tip60 acts as an adaptator to allow the recruitment and to stimulate the activity of specific ubiquitin ligase complex towards SRSF2. Importantly, we demonstrate that the deacetylase HDAC6 interacts with and stabilizes SRSF2 by a mechanism that requires its deacetylase domain. We also show that lysine 52 is a critical residue in this process. These results suggest that deacetylation of SRSF2 by HDAC6 could prevent its further ubiquitination and degradation. It is also known that high-affinity binding of HDAC6 to ubiquitin prevents the access of other cellular factors to poly-ubiquitin chains, thereby protecting ubiquitinated proteins from proteasomal degradation (Boyault *et al*, 2007b). Since we demonstrate that ubiquitin-binding domain of HDAC6 is required for SRSF2 accumulation, our results suggest that HDAC6 could bind an acetylated and ubiquitinated form of SRSF2 and prevent its further degradation. This could explain why SRSF2 still accumulates in cells overexpressing Tip60 in the presence of high amounts of HDAC6 (Figure 3F) and support a model in which the equilibrium between Tip60 and HDAC6 activities controls SRSF2 protein turn-over (Figure 7A). Further experiments are now needed to more thoroughly examine the relationship between SRSF2 and the components of the ubiquitination machinery.

Phosphorylation of SR proteins is essential for their nuclear import and is required for initiation of spliceosome assembly (Cao *et al*, 1997; Xiao and Manley, 1997). Conversely, dephosphorylation of SR proteins is critical for splicing catalysis once the spliceosome is assembled (Cao *et al*, 1997; Mermoud *et al*, 1992) and for several post-splicing events

(Sanford *et al*, 2005). Therefore, phosphorylation of SR proteins must be tightly regulated. Several SR protein kinases have been identified to date. The SRPK1 and SRPK2 kinases are among the best-characterized ones. They are detected in both the cytoplasm and the nucleus (Ding *et al*, 2006; Wang *et al*, 1998), and play an important role in nuclear import as well as in intranuclear localization of SR proteins (Gui *et al*, 1994; Yeakley *et al*, 1999). SRPKs are major kinases for SR proteins in mammalian cells (Hayes *et al*, 2006). Consistently, we provide evidence that both SRPK1 and SRPK2 proteins are the main regulators of SRSF2 phosphorylation in our cellular models. Moreover, we demonstrate that Tip60 decreases the phosphorylation of SRSF2 by a mechanism requiring its HAT activity, unraveling a close interplay between acetylation and phosphorylation signaling pathways. It has been recently proposed, in the same way that phosphorylation affects acetylation of a neighboring lysine, that acetylation might also regulate phosphorylation of adjacent or distant residues (Yang and Seto, 2008). In addition recent studies have shown that, while recognition of the RS domain of SRSF1 by a docking groove on SRPK1 is sufficient to initiate processive and directional phosphorylation, continued phosphorylation relies on the fine-tuning of contacts with RRM modules (Hagopian *et al*, 2008; Ngo *et al*, 2008). Based on these data, it might be speculated that acetylation of SRSF2 inside its RRM by Tip60 could interfere with SRPK-mediated phosphorylation. However, as HDAC6 as well as the mutation of lysine 52 into a non-acetylable residue do not modify SRSF2 phosphorylation (Edmond, unpublished results), acetylation is probably not involved in the inhibition of SRSF2 phosphorylation by Tip60. Consistent with such hypothesis, we provide further evidence that Tip60, but not its inactive catalytic mutant Tip60^{G380}, inhibits the nuclear translocation of both SRPK1 and SRPK2 kinases. These data indicate that an inappropriate sub-cellular distribution of SRPKs might mainly contribute to the negative effect of Tip60 on SRSF2 phosphorylation. It has been shown that the spacer sequence that splits the conserved kinases domains of SRPK1 and SRPK2 into

two blocks is involved in their partitioning between the cytoplasm and the nucleus (Ding *et al*, 2006). Whether Tip60 could target directly or indirectly this accessory domain to affect SRPKs nuclear accumulation remains to be determined. Of note, SRPK1 was identified as a putative acetylated protein by Choudhary and colleagues (Choudhary *et al*, 2009), thereby suggesting that Tip60 could at least acetylate SRPK1 to control its sub-cellular distribution. However, as signaling pathways involving the major molecular chaperone HSP90 (Zhong *et al*., 2009) or AKT (Jang *et al*., 2009) were recently found to control SRPK1/2 sub-cellular partitioning, additional experiments are required to explore the effects of Tip60 on these processes.

In agreement with an important role of SRSF2 during DNA Damage Response (DDR) (Merdzhanova *et al*, 2008), we finally demonstrate that SRSF2 is upregulated and required for cellular apoptosis in response to cisplatin treatment. Importantly we show that, in this context, SRSF2 accumulates in a phosphorylated/hypoacetylated form. Such post-translational modifications of SRSF2 are accompanied by a strong decrease of Tip60 mRNA and protein expression, as well as with the nuclear accumulation of both SRPK1 and SRPK2 kinases. The crucial role of Tip60 during DDR is now emerging (Squatrito *et al*, 2006) and high amounts of Tip60 protein have been correlated with cisplatin resistance in several human lung carcinoma cell lines (Miyamoto *et al*, 2008). Therefore, our results are consistent with a model in which cisplatin-induced apoptosis involves Tip60 downregulation, SRSF2 stabilization and SRSF2 phosphorylation (Figure 7B). In addition, as HDAC6 is required for SRSF2 stabilization (Figure 5E) as well as for apoptosis occurrence (data not shown) in this context, the Tip60/HDAC6 ratio would play a key role in the control of SRSF2 expression in response to cisplatin. Moreover, we also demonstrate that SRPK2 is absolutely required for SRSF2 phosphorylation and determines cell fate (growth arrest versus apoptosis). In contrast, SRPK1 appears to have opposite functions since its neutralization increases rather than inhibits

apoptosis. Of note, such a role of SRPK1 in resistance to cisplatin has been previously reported in pancreatic carcinoma (Hayes *et al*, 2006; Hayes *et al*, 2007). Therefore, and as recently suggested (Mathew *et al*, 2008), our data provide further evidence that each SRPK have both overlapping and independent functions. Furthermore, they support a critical role of SRPK2-mediated SRSF2 phosphorylation in the induction of apoptosis following cisplatin treatment.

To conclude, our study highlights a new acetylation/phosphorylation signaling network targeting the SRSF2 protein, to control cellular apoptosis in response to genotoxic stress. These data reinforce the connection between chromatin and splicing players, and open the way to further experiments aiming at thoroughly examine the role of such connection in the control of pre-mRNA splicing events.

Materials and Methods

Cell lines, cell treatments, plasmids and transfection

H358, H1299, H810, H69 human lung carcinoma cell lines and SAOS2 osteosarcoma cells were cultured as described (Salon *et al*, 2007; Merdzhanova *et al*, 2008). Stable inducible SRSF2 clones derived from H358 cells were obtained using a modified tetracyclin-regulated inducible expression system (Tet-On System, Clontech, Ozyme, Saint Quentin en Yvelines, France) as previously described (Salon *et al*, 2006). The parental and HDAC6-/- 3T3 cell lines, as well as the 3T3 cell lines derived from HDAC6-deficient cells re-expressing wild-type HDAC6 (+WT) or HDAC6 bearing mutations either in the two catalytic deacetylase domains (HD^m) or in the ZnF-UBP ubiquitin-binding domain (Ub^m) have been previously described (Boyault *et al*, 2007a). They were maintained in DMEM (GibcoBRL) supplemented with 10% (v/v) heat-inactivated FCS, 2mM L-glutamine and 100U/ml penicillin/streptomycin in 5% CO_2

at 37°C. Plasmids and reagents used in this study are described in details in the Supplementary Information.

Immunoprecipitation and western blotting experiments

Immunoprecipitation and western blotting experiments were performed as previously described (Salon *et al*, 2006). All the antibodies used in this study are described in the Supplementary Informations. Cellular fractionation experiments were performed as described in the Supplementary Materials.

***In vitro* acetylation assays**

Distinct cDNA encoding truncated forms of SRSF2 protein were fusionned in frame with GST by sub-cloning into pGEX-6P3 plasmid. Beads coated with GST, GST-SRSF2(1-60), GST-SRSF2(60-115), GST-SRSF2(115-221) or GST-SRSF1 fusion proteins were prepared according to the manufacturer's protocol (Bulk GST Purification module, Pharmacia Biotech). "*In vitro*" acetyltransferase assays were performed using 2 µg of each recombinant GST-SRSF2 fusion peptides, 1 µg of recombinant His-tagged $Tip60^{212-513}$ or His-tagged GCN5 protein and 0.05 µCi of [^{14}C]acetyl-CoA, as described previously (Leduc *et al*, 2006).

Transfection of siRNA oligonucleotides

The sequences designed to specifically target human *SRSF2*, *Tip60*, *HDAC6*, *srpk1* and *srpk2* RNAs are listed in the Supplementary Information. Cells were transfected with siRNA oligonucleotides duplex using Oligofectamine reagent according to the manufacturer's instructions (Invitrogen, Cergy Pontoise, France), excepted for H810 cells transfected with Hi-Perfect reagent (Qiagen). The cells were analyzed 72 hours post-transfection.

Quantitative RT-PCR and RT-PCR analyses

Quantitative RT-PCR was performed on Stratagene MX3005P apparatus (Agilent Technologies), as previously described (Van Den Broeck *et al*, 2008). The specific primers used for mRNA amplification are described in the Supplementary Information. RT-PCR analysis of caspase-8 splice variants was performed as described previously (Merdzhanova *et al*, 2008).

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References

Batsche E, Yaniv M, Muchardt C (2006) The human SWI/SNF subunit Brm is a regulator of alternative splicing. *Nat Struct Mol Biol* **13:** 22-29

Boyault C, Gilquin B, Zhang Y, Rybin V, Garman E, Meyer-Klaucke W, Matthias P, Muller CW, Khochbin S (2006) HDAC6-p97/VCP controlled polyubiquitin chain turnover. *EMBO J* **25:** 3357-3366

Boyault C, Zhang Y, Fritah S, Caron C, Gilquin B, Kwon SH, Garrido C, Yao T P, Vourc'h C, Matthias P, Khochbin S (2007a) HDAC6 controls major cell response pathways to cytotoxic accumulation of protein aggregates. *Genes Dev* **21:** 2172-2181

Boyault C, Sadoul K, Pabion M, Khochbin S (2007b) HDAC6, at the crossroads between cytoskeleton and cell signaling by acetylation and ubiquitination. *Oncogene* **26:** 5468-5476

Cao W, Jamison SF, Garcia-Blanco MA (1997) Both phosphorylation and dephosphorylation of ASF/SF2 are required for pre-mRNA splicing in vitro. *RNA* **3:** 1456-1467

Caron C, Boyault C, Khochbin S (2005) Regulatory cross-talk between lysine acetylation and ubiquitination: role in the control of protein stability. *Bioessays* **27:** 408-415

Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, Olsen JV, Mann M (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* **325:** 834-840

Ding JH, Zhong XY, Hagopian JC, Cruz MM, Ghosh G, Feramisco J, Adams JA, Fu XD (2006) Regulated cellular partitioning of SR protein-specific kinases in mammalian cells. *Mol Biol Cell* **17:** 876-885

Dino Rockel T, von Mikecz A (2002) Proteasome-dependent processing of nuclear proteins is correlated with their subnuclear localization. *J Struct Biol* **140:** 189-199

Graveley BR (2000) Sorting out the complexity of SR protein functions. *RNA* **6:** 1197-1211

Gui JF, Lane WS, Fu XD (1994) A serine kinase regulates intracellular localization of splicing factors in the cell cycle. *Nature* **369:** 678-682

Hagopian JC, Ma CT, Meade BR, Albuquerque CP, Ngo JC, Ghosh G, Jennings PA, Fu XD, Adams JA (2008) Adaptable molecular interactions guide phosphorylation of the SR protein ASF/SF2 by SRPK1. *J Mol Biol* **382:** 894-909

Hayes GM, Carrigan PE, Beck AM, Miller LJ (2006) Targeting the RNA splicing machinery as a novel treatment strategy for pancreatic carcinoma. *Cancer Res* **66:** 3819-3827

Hayes GM, Carrigan PE, Miller LJ (2007) Serine-arginine protein kinase 1 overexpression is associated with tumorigenic imbalance in mitogen-activated protein kinase pathways in breast, colonic, and pancreatic carcinomas. *Cancer Res* **67:** 2072-2080

Himeji D, Horiuchi T, Tsukamoto H, Hayashi K, Watanabe T, Harada M (2002) Characterization of caspase-8L: a novel isoform of caspase-8 that behaves as an inhibitor of the caspase cascade. *Blood* **99:** 4070-4078

Ianari A, Gallo R, Palma M, Alesse E, Gulino A (2004) Specific role for p300/CREB-binding protein-associated factor activity in E2F1 stabilization in response to DNA damage. *J Biol Chem* **279:** 30830-30835

Jang SW, Liu X, Fu H, Rees H, Yepes M, Levey A, Ye K (2009) Interaction of Akt-phosphorylated SRPK2 with 14-3-3 mediates cell cycle and cell death in neurons. J Biol Chem **284:** 24512-24525.

Johnson JM, Castle J, Garrett-Engele P, Kan Z, Loerch PM, Armour CD, Santos R, Schadt EE, Stoughton R, Shoemaker DD (2003) Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. *Science* **302:** 2141-2144

Kamachi M, Le TM, Kim SJ, Geiger ME, Anderson P, Utz PJ (2002) Human autoimmune sera as molecular probes for the identification of an autoantigen kinase signaling pathway. *J Exp Med* **196:** 1213-1225

Lai MC, Lin RI, Tarn WY (2003) Differential effects of hyperphosphorylation on splicing factor SRp55. *Biochem J* **371:** 937-945

Leduc C, Claverie P, Eymin B, Col E, Khochbin S, Brambilla E, Gazzeri S (2006) p14ARF promotes RB accumulation through inhibition of its Tip60-dependent acetylation. *Oncogene* **25:** 4147-4154

Legube G, Linares LK, Lemercier C, Scheffner M, Khochbin S, Trouche D (2002) Tip60 is targeted to proteasome-mediated degradation by Mdm2 and accumulates after UV irradiation. *EMBO J* **21:** 1704-1712

Liu HX, Zhang M, Krainer AR (1998) Identification of functional exonic splicing enhancer motifs recognized by individual SR proteins. *Genes Dev* **12:** 1998-2012

Logan IR, Sapountzi V, Gaughan L, Neal DE, Robson CN (2004) Control of human PIRH2 protein stability: involvement of TIP60 and the proteosome. *J Biol Chem* **279:** 11696-11704

Loomis RJ, Naoe Y, Parker JB, Savic V, Bozovsky MR, Macfarlan T, Manley JL, Chakravarti D (2009) Chromatin binding of SRp20 and ASF/SF2 and dissociation from mitotic chromosomes is modulated by histone H3 serine 10 phosphorylation. *Mol Cell* **33:** 450-461

Mathew R, Hartmuth K, Mohlmann S, Urlaub H, Ficner R, Luhrmann R (2008) Phosphorylation of human PRP28 by SRPK2 is required for integration of the U4/U6-U5 tri-snRNP into the spliceosome. *Nat Struct Mol Biol* **15:** 435-443

Merdzhanova G, Edmond V, De Seranno S, Van den Broeck A, Corcos L, Brambilla C, Brambilla E, Gazzeri S, Eymin B (2008) E2F1 controls alternative splicing pattern of genes involved in apoptosis through upregulation of the splicing factor SRSF2. *Cell Death Differ* **15:** 1815-1823

Mermoud JE, Cohen P, Lamond AI (1992) Ser/Thr-specific protein phosphatases are required for both catalytic steps of pre-mRNA splicing. *Nucleic Acids Res* **20:** 5263-5269

Miyamoto N, Izumi H, Noguchi T, Nakajima Y, Ohmiya Y, Shiota M, Kidani A, Tawara A, Kohno K (2008) Tip60 is regulated by circadian transcription factor clock and is involved in cisplatin resistance. *J Biol Chem* **283:** 18218-18226

Ngo JC, Giang K, Chakrabarti S, Ma CT, Huynh N, Hagopian JC, Dorrestein PC, Fu XD, Adams JA, Ghosh G (2008) A sliding docking interaction is essential for sequential and processive phosphorylation of an SR protein by SRPK1. *Mol Cell* **29:** 563-576

Sadoul K, Boyault C, Pabion M, Khochbin S (2008) Regulation of protein turnover by acetyltransferases and deacetylases. *Biochimie* **90:** 306-312

Salon C, Eymin B, Micheau O, Chaperot L, Plumas J, Brambilla C, Brambilla E, Gazzeri S (2006) E2F1 induces apoptosis and sensitizes human lung adenocarcinoma cells to death-

receptor-mediated apoptosis through specific downregulation of c-FLIP(short). *Cell Death Differ* **13:** 260-272

Salon C, Merdzhanova G, Brambilla C, Brambilla E, Gazzeri S, Eymin B (2007) E2F-1, Skp2 and cyclin E oncoproteins are upregulated and directly correlated in high-grade neuroendocrine lung tumors. *Oncogene* **26:** 6927-6936

Sanford JR, Ellis J, Caceres JF (2005) Multiple roles of arginine/serine-rich splicing factors in RNA processing. *Biochem Soc Trans* **33:** 443-446

Schwartz S, Meshorer E, Ast G (2009) Chromatin organization marks exon-intron structure. *Nat Struct Mol Biol* **16:** 990-995

Seigneurin-Berny D, Verdel A, Curtet S, Lemercier C, Garin J, Rousseaux S, Khochbin S (2001) Identification of components of the murine histone deacetylase 6 complex: Link between acetylation and ubiquitination signaling pathways. *Mol Cell Biol* **21:** 8035-8044

Sims RJ 3rd, Millhouse S, Chen CF, Lewis BA, Erdjument-Bromage H, Tempst P, Manley JL, Reinberg D (2007) Recognition of trimethylated histone H3 lysine 4 facilitates the recruitment of transcription postinitiation factors and pre-mRNA splicing. *Mol Cell* **28:** 665-676

Squatrito M, Gorrini C, Amati B (2006) Tip60 in DNA damage response and growth control: many tricks in one HAT. *Trends Cell Biol* **16:** 433-442

Stamm S (2008) Regulation of alternative splicing by reversible protein phosphorylation. *J Biol Chem* **283:** 1223-1227

Sureau A, Gattoni R, Dooghe Y, Stevenin J, Soret J (2001) SRSF2 autoregulates its expression by promoting splicing events that destabilize its mRNAs. *Embo J* **20:** 1785-1796

Tilgner H, Nikolaou C, Althammer S, Sammeth M, Beato M, Valcarcel J, Guigo R (2009) Nucleosome positioning as a determinant of exon recognition. *Nat Struct Mol Biol* **16:** 996-1001

Van Den Broeck A, Brambilla E, Moro-Sibilot D, Lantuejoul S, Brambilla C, Eymin B, Khochbin S, Gazzeri S (2008) Loss of histone H4K20 trimethylation occurs in preneoplasia and influences prognosis of non-small cell lung cancer. *Clin Cancer Res* **14:** 7237-7245

Wang HY, Lin W, Dyck JA, Yeakley JM, Songyang Z, Cantley LC, Fu XD (1998) SRPK2: a differentially expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells. *J Cell Biol* **140:** 737-750

Wu JY, Maniatis T (1993) Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. *Cell* **75:** 1061-1070

Xiao SH, Manley JL (1997) Phosphorylation of the ASF/SF2 RS domain affects both protein-protein and protein-RNA interactions and is necessary for splicing. *Genes Dev* **11:** 334-344

Yang XJ, Seto E (2008) Lysine acetylation: codified crosstalk with other posttranslational modifications. *Mol Cell* **31:** 449-461

Yeakley JM, Tronchere H, Olesen J, Dyck JA, Wang HY, Fu XD (1999) Phosphorylation regulates in vivo interaction and molecular targeting of serine/arginine-rich pre-mRNA splicing factors. *J Cell Biol* **145:** 447-455

Zhong XY, Ding JH, Adams JA, Ghosh G, Fu XD (2009) Regulation of SR protein phosphorylation and alternative splicing by modulating kinetic interactions of SRPK1 with molecular chaperones. *Genes Dev* **23:** 482-495

Figure legends

Figure 1 SRSF2 is acetylated on its lysine 52 (K52) residue by Tip60. (**A**) SAOS2 cells were transfected for 48 h with a HA-tagged SRSF2 expression vector. Whole-cellular extracts were subjected to immunoprecipitation with either anti-HA antibody or irrelevant immunoglobulin (IgG) as a negative control, followed by immunoblotting with anti-acetyl-lysine (Ac-K) or anti-HA antibody. (**B**) Endogenous acetylated SRSF2 protein was detected from H69 nuclear-enriched extract after immunoprecipitation of acetylated proteins with an anti-acetyl-lysine (left panel) or anti-SRSF2 (right panel) antibody and immunodetection using anti-SRSF2 or anti-acetyl-lysine antibody, respectively. (**C**) Representation of recombinant truncated GST-SRSF2 fusion proteins and recombinant His-tagged $Tip60^{212-513}$ and GCN5 acetyltransferases. (**D**) Purified GST-SRSF2 fusion proteins were incubated with recombinant His-tagged $Tip60^{212-513}$ or hGCN5 in the presence of [^{14}C]acetyl-CoA. Acetylation was revealed after autoradiography (upper panel). Equivalent amounts of various recombinant proteins were assessed by Coomassie staining (lower panel). (**E**) H1299 cells were transfected for 48 h with either *mismatch* or *Tip60* siRNAs. Total cellular extracts were subjected to immunoprecipitation with an anti-Ac-K (upper panel) or an anti-SRSF2 (middle panel) antibody followed by immunoblotting with anti-SRSF2, anti-SRSF1 or anti-acetyl-lysine antibody as indicated. Neutralization of Tip60 was controlled by RT-QPCR (lower panel). (**F**) GST-SRSF2 (1-60) wild-type (WT) or point mutants (K17R; K52R) recombinant proteins were incubated with [^{14}C]acetyl-CoA in the presence (+) or absence (-) of recombinant His-tagged $Tip60^{212-513}$. Analysis of SRSF2 acetylation was performed as in **D**. K17R and K52R: substitution of the indicated lysine by arginine (lower panel).

Figure 2 Acetylation by Tip60 controls SRSF2 turn-over. (**A**) H358 and H1299 cell lines were transfected for 72 h with *mismatch* or *Tip60(2)* siRNAs. SRSF2, SRSF1 and Tip60 protein levels were analyzed by western blotting. Actin was used as a loading control (upper panel). *SRSF2* mRNA levels were quantified by RT-QPCR. *G3pdh* was used as an internal control (lower panel). (**B**) H1299 cells were co-transfected for 72 h with *mismatch* or *Tip60(2)* siRNAs in the presence or absence of either wild-type (WT) HA-Tip60 or mutant (G380) HA-Tip60^{G380} with impaired HAT domain. SRSF2 protein expression was assessed by western blotting (upper panel). Neutralization or overexpression of Tip60 was verified by RT-QPCR (lower panel). (**C**) HA-tagged SRSF2 protein was co-expressed for 36 h in H1299 cells with either wild-type (WT) HA-Tip60 or mutant (G380) HA-Tip60^{G380}, and treated (+) or not (-) for 18 additional hours with MG132. Western blot analysis using anti-HA antibody is presented (upper panel). SRSF2 and actin signal intensities were quantified using the ImageJ software and the relative densitometric areas for SRSF2-HA were determined according to actin signal in each condition (lower panel). (**D**) H1299 cells were transfected for 36 h with plasmid encoding either wild type (WT) HA-Tip60 or mutant (G380) HA-Tip60^{G380} protein and treated (+) or not (-) for 18 additional hours with MG132. SRSF2 protein level was analyzed by western blot. (upper panel). The relative densitometric areas for SRSF2 were determined as in C (lower panel). (**E**) H1299 cells were transfected for 36 h with plasmid encoding lysine 52 mutant (K52R) HA-tagged SRSF2 protein in the presence or absence (-) of either wild type (WT) HA-Tip60 or mutant (G380) HA-Tip60^{G380} and treated (+) or not (-) for 18 additional hours with MG132 as indicated. SRSF2(K52R) and Tip60 proteins were detected by western blotting using anti-HA antibody (upper panel) and the relative densitometric areas for SRSF2(K52R) were determined (lower panel). (**F**) H1299 cells were transfected for 48 h with HA-SRSF2 WT or K52R expression vector then treated with cycloheximide (CHX) for the indicated times. Whole cellular extracts were subjected to western blotting using anti-HA

antibody (upper panel). HA-SRSF2 densitometric signals were normalized to actin. A value of 1 was arbitrarily assigned to the signal obtained at zero time of CHX treatment (lower panel).

Figure 3 HDAC6 acts as a positive regulator of SRSF2 protein level by counteracting Tip60-mediated effects. (**A**) H1299 cells were co-transfected for 48 h with HA-tagged SRSF2 and Flag-tagged HDAC6 expression vectors. SRSF2 protein level was analyzed by western blotting using an anti-HA antibody. (**B**) H358 and H810 cells were transfected for 72 h with *mismatch* or *HDAC6* siRNAs. Expression of SRSF2 and SRSF1 proteins was studied by western blotting. (**C**) H1299 cells were transfected for 72 h with either *mismatch* or *HDAC6* siRNAs. Total cellular extracts were subjected to immunoprecipitation with anti-Ac-K52 SRSF2 antibody followed by immunoblotting with anti-SRSF2 antibody (upper panel). H1299 cells were transfected for 48 h with HA-tagged SRSF2 WT or K52R expression vector, in the presence (+) or absence (-) of Flag-tagged HDAC6 and subjected to western blot analysis for detection of HA-SRSF2 expression. The relative densitometric areas for HA-SRSF2 were determined according to actin signal (middle panel). H1299 cells were co-transfected for 48 h with HA-tagged SRSF2 WT or K52R expression vector, in the presence or absence of *mismatch* or *HDAC6* siRNA as indicated and subjected to western blot analysis for detection of HA-SRSF2 or HDAC6 expression (lower panel). (**D**) Whole cellular extracts derived from 3T3 cell lines wild-type (WT), HDAC6-/- (KO) or HDAC6-/- re-expressing the wild-type HDAC6 (+WT) were used to analyze SRSF2 protein level (upper panel). One of the HDAC6-deficient clones was used to establish new lines re-expressing wild-type HDAC6 (+WT) or a catalytically dead (+HD^m) or a non-ubiquitin-binding mutant of HDAC6 (Ub^m) (Boyault *et al*, 2007a). Whole cellular extracts were used to study SRSF2 expression (lower panel). (**E**) Same cells were treated (+) or not (-) with proteasome inhibitor MG132 for 18 hours and SRSF2 expression was analyzed by western blot. (**F**) HA-tagged SRSF2 WT or K52R protein was co-expressed for 48 h with HA-tagged Tip60 protein in H1299 cells, in the presence of increasing amount of Flag-

tagged HDAC6 expression vector. SRSF2 protein expression was detected by western blotting using an anti-HA antibody.

Figure 4 Tip60 down-regulates SRSF2 phosphorylation by preventing SRPK1/2 nuclear translocation. (**A**) H1299 cells were co-transfected for 48 h with HA-tagged SRSF2 and either HA-Tip60 WT or HA-Tip60^{G380} expression vectors and treated for 18 h with MG132. SRSF2 phosphorylation was studied by western blotting using either mAb104 or phospho-SRSF2 (P-SRSF2) antibody. (**B**) H1299 cells were transfected for 48 h with plasmid encoding HA-tagged SRSF2 protein in the presence or absence of HA-tagged Tip60 expression vector and treated with MG132 18h before harvesting. Cellular extracts were subjected to immunoprecipitation with an anti-HA antibody followed by immunoblotting with an anti-phospho-SRSF2 (P-SRSF2) or anti-SRSF2 antibody. (**C**) H358 cells were transfected for 72 h with either *mismatch* or *Tip60(1)* siRNAs. SRSF2 phosphorylation was studied by western blotting using a specific P-SRSF2 antibody (left panel). Neutralization of Tip60 was controlled by RT-QPCR (right panel). (**D**) HA-tagged SRSF2 protein was co-expressed in H1299 cells with HA-tagged Tip60 protein. Total cellular extracts (upper panel) or cytoplasmic (C) and nuclear (N) extracts (lower panel) were subjected to western blot. Anti-α-tubulin and anti-histone H3 antibodies were used to assess the fractionation efficiency. (**E**) H810 cells were transfected for 72 h with *mismatch* or *Tip60(1)* siRNAs. Total cellular extracts were immunoblotted with anti-SRPK1 or anti-SRPK2 antibody (upper panel). Cytoplasmic (C) and nuclear (N) extracts were subjected to immunoblotting using the indicated antibodies. (lower panel). (**F**) H810 cells were transfected for 72 h with *mismatch* or *Tip60(2)* siRNA. Immunolocalization of either SRPK1 or SRPK2 protein (green) was visualized by immunofluorescence. DAPI staining (blue) was used to counterstain nuclei.

Figure 5 SRSF2 accumulates in a hypoacetylated/phosphorylated form in response to cisplatin treatment and is required for apoptosis. (**A**, **B**) H358 cells were transfected for 48 h with either *mismatch* or *SRSF2* siRNAs and treated (+) or not (-) for 24 additional hours with cisplatin (100 µM). (**A**) Western blot analysis was performed using anti-SRSF2 or anti-active caspase-3 antibody (left panel). Apoptosis was quantified by flow cytometry analysis of active caspase-3 in untreated (grey bars) or treated (black bars) cells (right panel). (**B**) RT-PCR analysis using specific primers of *caspase-8* splice variants. *Gapdh* was used as an internal control (left panel). Densitometric signals were quantified using the Image J software and the relative ratio caspase-8L/caspase-8a was calculated in either untreated (grey bars) or treated (black bars) cells (right panel). (**C**) H810 cells were treated or not for 24 h with 100 µM cisplatin. Nuclear-enriched extracts were prepared and immunoprecipitation of acetylated proteins was performed using an anti-acetyl lysine antibody followed by immunoblotting using either an anti-SRSF2 or anti-E2F1 antibody. (**D**) H810 cells were treated or not for 24 h with 100 µM cisplatin and subjected to western blot analysis (left panel). Tip60 transcript level was quantified by RT-QPCR (right panel). (**E**) H358 cells were transfected for 48 h with *mismatch* or *HDAC6* siRNAs and treated (+) or not (-) for 24 additional hours with 100 µM cisplatin. Western blot analysis was performed for the detection of SRSF2 or HDAC6 protein (upper panel). Wild-type or HDAC6 knock-out 3T3 cells were treated (+) or not (-) with 100 µM cisplatin for 24 hours and SRSF2 protein level was assessed by immunoblotting (lower panel). (**F**) Total cellular extracts were obtained from H358 and H1299 cells treated or not for 24 h with 100 µM cisplatin. P-SRSF2 and SRSF2 protein levels were analyzed by western-blotting. (**G**) Cytoplasmic and nuclear extracts from H810 cells treated or not for 24 h with 100 µM cisplatin were subjected to western blotting.

Figure 6 SRPK2 but not SRPK1 is required for SRSF2 phosphorylation and apoptosis in response to cisplatin. (**A**, **B**) H358 cells were transfected for 48 h with either *mismatch*, *SRPK1*

or *SRPK2* siRNAs and treated (+) or not (-) for 24 additional hours with 100 µM cisplatin. (**A**) Western blot analyses of the indicated proteins (left panel). Apoptosis was quantified in untreated (grey bars) or treated (black bars) cells by analysis of caspase-3 activation by flow cytometry (right panel). (**B**) RT-PCR analyses of *caspase-8* splice variants. *Gapdh* was used as an internal control (left panel). The relative ratio caspase-8L/caspase-8a was calculated in either untreated (grey bars) or treated (black bars) cells after densitometric quantification of the signals (right panel). (**C**) H358 cells were transfected for 48 h with *mismatch* or *SRPK2* siRNAs and treated or not for 24 additional hours with 100 µM cisplatin. Cell cycle distribution was analyzed by flow cytometry. Percentages of cells in the different phases are indicated.

Figure 7 A model for the roles of Tip60, HDAC6, SRPK1 and SRPK2 proteins in the control of SRSF2 protein in both unstressed (**A**) and genotoxic stressed (**B**) conditions. (**A**) In unstressed cells, Tip60 acetylates SRSF2 on its lysine 52 residue and prevents SRPK1 and SRPK2 nuclear localization. SRSF2 accumulates in a hyperacetylated/hypophosphorylated form that is subjected to proteasomal degradation. This effect is counterbalanced by the HDAC6 deacetylase which positively controls SRSF2 protein level by deacetylating SRSF2 and preventing its proteasomal degradation. (**B**) Upon cisplatin treatment, Tip60 protein level strongly decreases leading to the nuclear accumulation of both SRPK1 and SRPK2 kinases, to the stabilization of SRSF2 in a hypoacetylated/phosphorylated form by a mechanism involving HDAC6, and to the induction of apoptosis. In treated cells, both SRPK1 and SRPK2 kinases appear to act in an opposite way. SRPK2 phosphorylates SRSF2 and is absolutely required for apoptosis induction. Both SRSF2 and SRPK2 proteins control the splicing pattern of *caspase-8* in favor of its pro-apoptotic splice variant. In contrast, SRPK1 acts as an anti-apoptotic factor since its knock-down enhances apoptosis upon exposure to cisplatin. In the absence of SRPK2,

apoptosis is blocked and treated cells accumulates in G_2/M phase indicating that SRPK-mediated SRSF2 phosphorylation controls cell fate decision.