Oncogene (2013), 1–9

www.nature.com/onc

ORIGINAL ARTICLE

The transcriptional co-activator SND1 is a novel regulator of alternative splicing in prostate cancer cells

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Splicing abnormalities have profound impact in human cancer. Several splicing factors, including SAM68, have pro-oncogenic functions, and their increased expression often correlates with human cancer development and progression. Herein, we have identified using mass spectrometry proteins that interact with endogenous SAM68 in prostate cancer (PCa) cells. Among other interesting proteins, we have characterized the interaction of SAM68 with SND1, a transcriptional co-activator that binds spliceosome components, thus coupling transcription and splicing. We found that both SAM68 and SND1 are upregulated in PCa cells with respect to benign prostate cells. Upregulation of SND1 exerts a synergic effect with SAM68 on exon v5 inclusion in the *CD44* mRNA. The effect of SND1 on *CD44* splicing required SAM68, as it was compromised after knockdown of this protein or mutation of the SAM68-binding sites in the *CD44* pre-mRNA. More generally, we found that SND1 promotes the inclusion of *CD44* variable exons by recruiting SAM68 and spliceosomal components on *CD44* pre-mRNA. Inclusion of the variable exons in *CD44* correlates with increased proliferation, motility and invasiveness of cancer cells. Strikingly, we found that knockdown of SND1, or SAM68, reduced proliferation and migration of PCa cells. Thus, our findings strongly suggest that SND1 is a novel regulator of alternative splicing that promotes PCa cell growth and survival.

Oncogene advance online publication, 2 September 2013; doi:10.1038/onc.2013.360

Keywords: alternative splicing; SND1; SAM68; *CD44*; prostate cancer

INTRODUCTION

Nuclear processing of pre-mRNAs requires tightly regulated steps that ultimately yield a mature and functional mRNA. Splicing is the step that insures the removal of long non-coding sequences (introns) from the pre-mRNA and the joining of the exons. This phenomenon is driven by a large macromolecular complex, the spliceosome, composed of five small nuclear ribonucleoproteins (snRNPs) and over 200 auxiliary proteins.[1] In higher eukaryotes, a large number of exons can be alternatively spliced to yield different transcripts from a single gene, thereby increasing the coding potential of the genome.[2,3] Indeed, the large majority of multi-exon human genes undergo alternative splicing (AS) to produce at least two mRNA variants.[4,5] As regulation of AS profoundly influences physiological and pathological processes,[3,6] the full comprehension of the molecular mechanisms regulating this step of pre-mRNA processing is of fundamental importance.

Splicing is physically and functionally coupled to transcription.[7–10] Two models have been proposed for how transcription might affect changes in AS patterns. The 'recruitment model' suggests that the transcription apparatus physically interacts with splicing regulators, thereby affecting splicing decisions. The C-terminal domain (CTD) of the largest subunit of the RNA polymerase II (RNAPII) has a central role in this coupling process by favoring the recruitment of RNA processing factors on the nascent transcripts.[9,10] Such regulation may also act bidirectionally, as splicing factors bound to complexes on nascent pre-mRNA can also modulate transcription.[11,12] For instance, the splicing factor SRSF2 (SC35) was shown to influence the post-translational modification of RNAPII and, as a consequence, its elongation rate.[12] A second model, known as 'kinetic model', proposes that changes in RNAPII elongation rate modulate exon inclusion by altering the availability of suboptimal splice sites.[13] In particular, a slow elongation rate allows the splicing factors to recognize weak splice site on the nascent transcripts, thus favoring the inclusion of variable exons in the mature mRNA. On the contrary, a faster elongation rate is often associated with the skipping of weak exons.[7,8,10]

The splicing machinery is an important target of misregulation in cancer.[14] In particular, changes in expression and/or activity of splicing factors and RNA-binding proteins (RBPs) correlate with cancer development, progression and response to therapy. RBPs are essential factors in RNA metabolism and their aberrant expression or regulation profoundly affects the gene expression profile of cancer cells.[14–16] An example of RBP that is upregulated in several human tumors is SAM68 (Src-associated in mitosis of 68 kDa), a member of the STAR (Signal Transduction Activator of RNA metabolism) family of RBPs.[17] SAM68 is involved in regulating several aspects of RNA processing, such as transcription,[18–21] AS[22–25] and translation of cellular mRNAs.[26–28] Moreover, SAM68 displays pro-oncogenic functions and is frequently upregulated in human cancer types,[17] including prostate cancer (PCa), wherein it supports cell proliferation and survival to genotoxic stresses.[29,30]

In spite of the increasing number of studies reporting a role of SAM68 in human cancer,[17,31] the mechanism(s) of action of this

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Received 5 March 2013; revised 3 June 2013; accepted 5 July 2013

protein in neoplastic cells is still largely unknown. As the activity and subcellular localization of SAM68 is influenced by its interaction with other proteins,[32,33] in the present work we set out to identify proteins that may affect SAM68 activity in PCa cells. Co-immunoprecipitation and mass spectrometry analyses allowed us to identify SND1 (Tudor-SN; p100) as a novel SAM68-interacting protein. SND1 is an ubiquitous protein and it is mainly known as a transcriptional co-activator. It interacts with several transcription factors and acts as a bridge between them and the transcriptional apparatus.[34,35] Furthermore, it interacts with the snRNPs and accelerates the kinetics of spliceosome assembly, thereby facilitating pre-mRNA splicing.[36,37] SND1 is also a component of the RNA-induced silencing complex that regulates RNAi-mediated gene silencing.[38] With regard to our study, recent evidence has suggested a positive role of SND1 in carcinogenesis. SND1 is overexpressed in human tumors, such as breast,[39,40] colon[41] and hepatocellular carcinomas.[42] Interestingly, similar to SAM68, SND1 is also upregulated in PCa, where the expression of the protein correlates with poor prognosis.[43] In this study, we characterized the interaction between SAM68 and SND1 biochemically and functionally and found that SND1 regulates AS of the variable exons of the CD44 receptor in a SAM68-dependent manner. Moreover, we show that the upregulation of SND1, similar to SAM68, promotes PCa cell migration. Thus, our findings identify a novel function for SND1 that might be critical in promoting PCa progression and metastasis.

RESULTS

Identification of SND1 as a novel SAM68-interacting protein

To identify SAM68-interacting proteins that could potentially affect its function in PCa cells, the endogenous protein was immunoprecipitated from LNCaP cell extracts. The bound proteins were subjected to SDS–PAGE, visualized with Silver Staining and identified using mass spectrometry (Figure 1a). This approach confirmed some previously described SAM68 interactions, such as hnRNP A1[23] and K,[44] nucleolin, heat shock 70-kDa protein 5 and eIF4B;[45] however, it also yielded many new interacting proteins that are potentially interesting for the function of SAM68 (Figure 1b, Supplementary Table 1). PSF, for example, is a splicing regulator that associates with the androgen receptor and regulates its transcriptional activity,[46] similar to SAM68,[21] whereas the translation initiation factor eIF3 and the ribosomal proteins L7, S3, S4 and S7 might be involved in the mechanism of SAM68-dependent regulation of mRNA translation.[26–28] In this

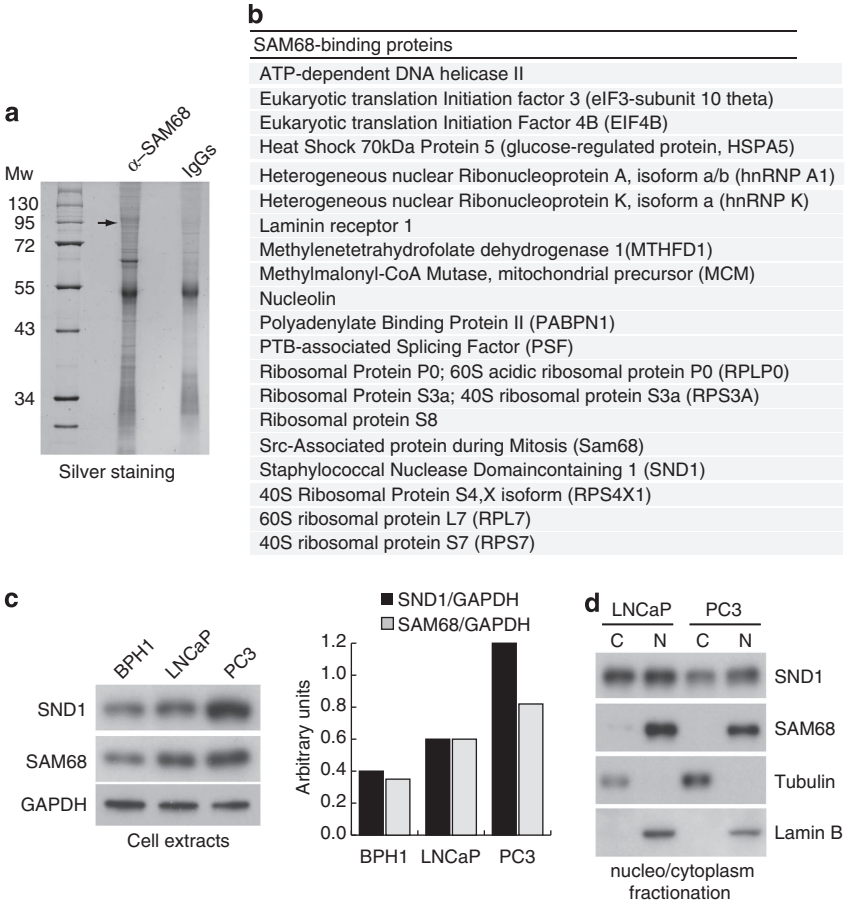

Figure 1. Identification of SND1 as a novel SAM68-associated factor upregulated in PCa cells. (**a**) Silver staining of a co-immunoprecipitation experiment in LNCaP cells. Total cell extracts were immunoprecipitated with an anti-SAM68 antibody or control IgGs and subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Interacting proteins were detected using Silver Staining and identified using mass spectrometry. Molecular weight marker was loaded in the first lane. The arrow on the image indicates the band corresponding to SND1 (running at ~100 kDa). (**b**) List of SAM68-interacting proteins obtained from the immunoprecipitation experiment described in (**a**). (**c**) Western blot analysis showing the expression of SAM68 and SND1 in BPH1, LNCaP and PC3 cells. The bar graph represents the densitometric analysis of the expression levels expressed as the ratio of SAM68 or SND1 intensity versus GAPDH intensity used as loading control. (**d**) Characterization of the distribution of SAM68 and SND1 using cell fractionation. Cytoplasmic (c) and nuclear (n) extracts were obtained from LNCaP and PC3 cells and analyzed using western blot. The efficiency of fractionation was verified by staining Tubulin and Lamin B as cytoplasmic and nuclear markers, respectively.

work, we have focused our attention on SND1 (Figure 1a), a transcriptional regulator that is also implicated in mRNA processing.[36,37]

First, by comparing its expression levels in PCa cells (LNCaP and PC3) with respect to cells derived from a benign prostate hyperplasia (BPH1), we found that SND1 was expressed at higher levels in PCa cells, similar to SAM68 (Figure 1c). Nucleus/cytoplasm fractionations of LNCaP and PC3 cell extracts indicated that SND1 is localized in both the nucleus and the cytoplasm (Figure 1d), consistent with its function in transcription, RNA interference and mRNA stability. Importantly, a substantial fraction of SND1 colocalizes in the nuclear fraction (N) together with SAM68 in both cell types (Figure 1d). Moreover, sucrose gradient centrifugation of nuclear extracts obtained from PCa cells showed that SND1 and SAM68 display a similar distribution profile, suggesting that the two proteins could be part of the same nuclear complexes in these cells (Supplementary Figure S1A).

To confirm the interaction between SAM68 and SND1, both proteins were independently immunoprecipitated from PCa cells. SND1 was efficiently co-immunoprecipitated with the endogenous SAM68 from LNCaP and PC3 nuclear extracts (Figures 2a and b). The complex formation was also detected when the experiment was performed reciprocally by immunoprecipitating SND1 from PC3 nuclear extracts (Figure 2c). Lastly, the interaction was tested by expressing recombinant proteins in HEK293T cells, which confirmed that Myc-SAM68 could be efficiently co-immunoprecipitated with FLAG-SND1 (Figure 2d) and by *in vitro* pull-down of the endogenous proteins from LNCaP cells with purified GST-SAM68 or GST-SND1 (Supplementary Figure S2 Notably, treatment of LNCaP cells with androgens or phorbol ester did not affect this interaction (Supplementary Figures S1B and C), suggesting that SND1 and SAM68 physically interact also under basal conditions. Collectively, these results point to SND1 as a particularly interesting candidate for the regulation of SAM68 function in PCa cells.

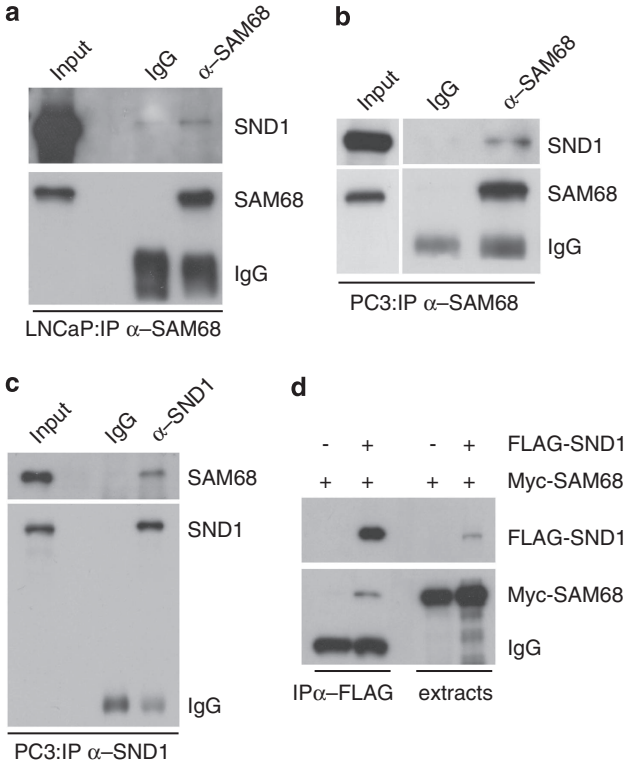

Figure 2. SND1 associates with SAM68 in PCa cells. (**a**–**c**) Western blot analyses of the co-immunoprecipitations between endogenous SAM68 and SND1. Nuclear extracts from the indicated cell lines were immunoprecipitated with nonspecific IgGs, anti-SAM68 (**a** and **b**) or anti-SND1 (**c**) antibodies. (**d**) HEK293T cells transfected with Myc-SAM68 alone or in combination with FLAG-SND1 were collected and the interaction between the two proteins was evaluated by co-immunoprecipitation using the anti-FLAG antibody; samples were resolved with SDS–PAGE and analyzed with western blot with anti-Myc and anti-FLAG antibodies.

SND1 cooperates with SAM68 in favoring CD44 exon v5 inclusion

Given the proposed role of SND1 in mRNA splicing,[36,37] we asked whether it could modulate the splicing activity of SAM68. As model system, we used *CD44*, for its relevance in cancer as the inclusion of its variable exons correlates with tumor development and metastasis.[14] SAM68 is known to promote the inclusion of exon v5 in *CD44*.[22,47,48] To test whether SND1 modulates the splicing activity of SAM68, we performed a splicing assay by transfecting the reporter pETv5 minigene (Figure 3a) with suboptimal amounts of FLAG-SND1 and GFP-SAM68. As expected, SAM68 significantly induced CD44 exon v5 inclusion at this dose (Figure 3b). Notably, transfection of SND1 alone also exhibited a mild effect on exon v5 inclusion (Figure 3b), which could be increased in a dose-dependent manner (Supplementary Figure S3). Furthermore, co-expression of SAM68 and SND1 synergistically enhanced exon v5 inclusion (Figure 3b). To obtain a more quantitative measure of the splicing activity, we carried out a splicing assay using pETv5 luciferase splice reporter construct[22] in HEK293T cells. This assay confirmed that suboptimal doses of SAM68 or SND1 alone mildly enhanced exon v5 inclusion, whereas their co-expression strongly increased it (Supplementary Figure S4). We also observed that exon v5 inclusion in the presence of suboptimal amount of GFP-SAM68 was enhanced by SND1 in a dose-dependent manner (Figure 3c), indicating a cooperation between the two proteins in the regulation of this splicing event. These results suggest that SND1 can act as a positive regulator of *CD44* AS.

SAM68 is required for the effect of SND1 on CD44 v5 exon inclusion

To test whether the effect of SND1 on *CD44* AS required the endogenous SAM68, we overexpressed the protein in PC3 cells stably silenced for SAM68. Similarly to what was observed in HEK293T cells (Supplementary Figure S3), SND1 stimulated the inclusion of exon v5 from the minigene in a dose-dependent manner in control PC3 cells (pLKO; Figure 3d). By contrast, its effect was notably reduced in cells depleted of SAM68 (pLKO-siSAM68; Figure 3d), indicating that SAM68 is required for the effect of SND1 on splicing.

SAM68 binds to sequences within exon v5 and in the upstream intron.[22,48] We took advantage of a pETv5 luciferase reporter gene in which both the exonic (L/CC mutant) and intronic (A/C mutant) binding sites are mutated[48] to confirm the requirement of SAM68 for the effect of SND1 on exon v5 splicing. Similar to what was observed in cells depleted of SAM68, mutation of both binding sites in the *CD44* minigene strongly decreased exon v5 inclusion mediated by FLAG-SND1 with respect to the effect exerted with the wild-type construct (Figure 3e).

Collectively, these experiments indicate that SAM68 is required for the effect of SND1 on *CD44* exon v5 inclusion.

SND1 expression affects the inclusion of variable exons in the endogenous CD44 transcript

Having established that SND1 is able to regulate exon v5 inclusion from a splicing reporter minigene, we next tested its activity on the endogenous *CD44* gene. The human gene contains nine variable exons (v2–v10) interposed between two sets of constitutive exons (c1–c5 and c6–c9)[49] (Figure 4A). We analyzed the expression of *CD44* splice variants in PC3 cells stably depleted of

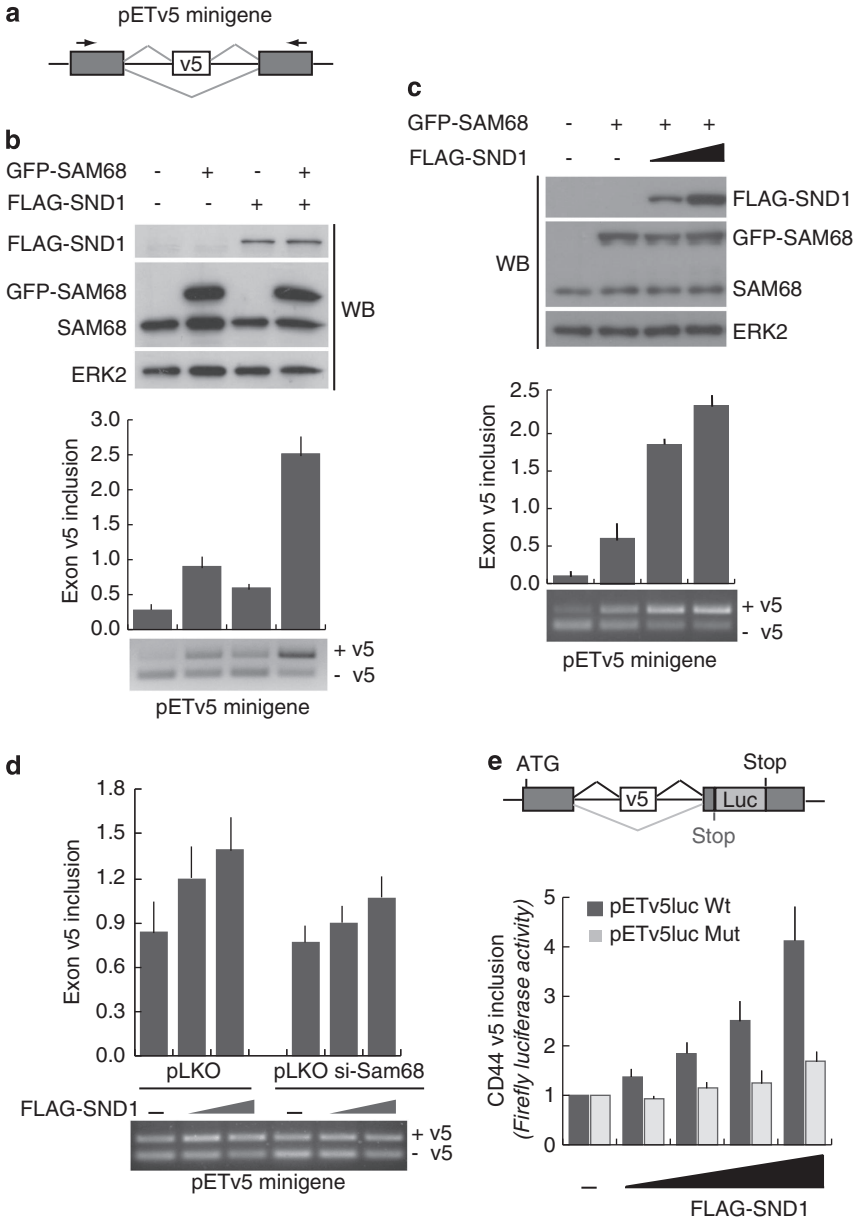

Figure 3. SND1 regulates CD44 exon v5 splicing in a SAM68-dependent manner. (**a**) Scheme of the pETv5 minigene used in panels (**b–d**).[22] (**b**) Splicing assay in HEK293T cells transfected with GFP-SAM68 or FLAG-SND1, either separately or together, and the pETv5 minigene. (**c**) Splicing assay in the same cells overexpressing GFP-SAM68 alone or in combination with increasing amount of FLAG-SND1 together with the pETv5 minigene. Western blot and densitometric analyses of the splicing assays are shown. (**d**) RT–PCR analysis of the *in vivo* splicing assay with the pETv5 minigene performed in control pLKO and pLKO si-SAM68 PC3 cells in presence of increasing amounts of SND1 protein. The bar graph indicates the levels of exon v5 inclusion. (**b–d**) All densitometric analyses of the splicing assays represent mean ± s.d. of three independent experiments. (**e**) Scheme of the pETv5 luciferase reporter construct. Luciferase assay in HEK293T cells transfected with the pETv5luc construct, wild type (black bars) or mutated (gray bars) for the SAM68-binding sites, Renilla luciferase plasmid (used as internal transfection control) and increasing amount of FLAG-SND1. Bar graph indicates the normalized luciferase activity ± s.d. from three independent transfections.

SND1 (si-SND1). Conventional RT–PCR analysis of the variable exons using primers in v2 and v10 indicated that depletion of SND1 reduced the *CD44* isoform containing all variant exons (Figure 4b). Interestingly, we observed that SND1 knockdown preferentially affected the upstream variable exons (v2–v5) with respect to those located downstream in the *CD44* gene (v8–v10; Figure 4b). On the other hand, amplification of constitutive exons (c6–c7) showed that SND1 did not affect the overall expression levels of *CD44* (Figure 4b). Quantitative RT–PCR (qPCR) analysis confirmed that the expression of upstream variable exons (v4, v5 and v7) was significantly decreased after the knockdown of SND1, whereas the inclusion of the downstream variable exons (v8–v10) and constitutive exons was not affected (Figure 4c). Consistent with the effect on splicing, chromatin immunoprecipitation experiments showed that SND1 is present within the coding region of *CD44*, with a moderate enrichment near the most-affected variable exons (Supplementary Figure S5A). As previously observed in HeLa cells,[47] silencing of SAM68 in PC3 cells reduced the inclusion of most of variable exons (Figure 4d). However, exons v4, v5 and v7, which were also sensitive to SND1 depletion,

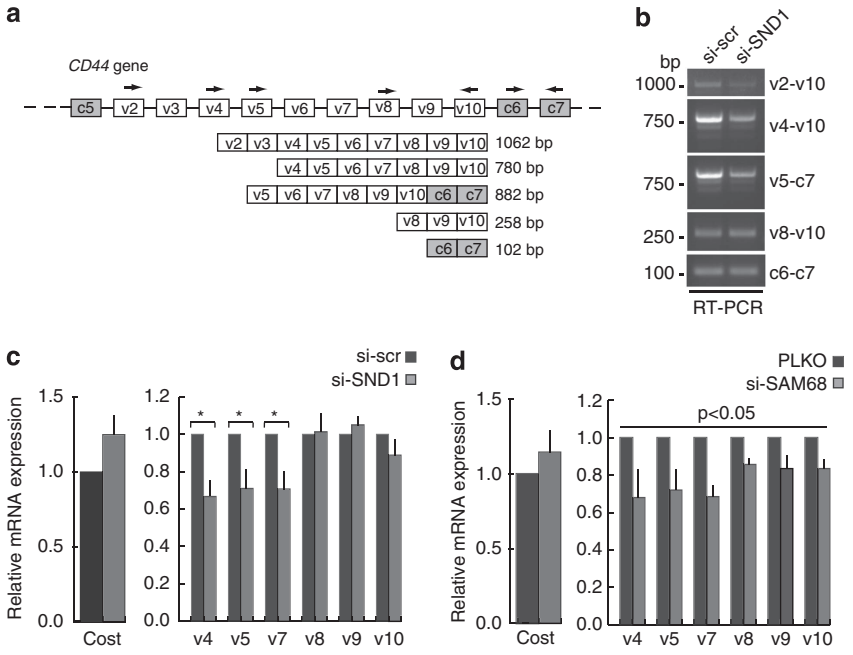

Figure 4. Effect of SND1 on the CD44 variable exon inclusion. (**a**) Schematic representation of the *CD44* gene; constant and variable exons are shown in gray and white, respectively. Arrows indicate the primers used in RT–PCR. Amplified sequences are shown below the gene map with the respective amplicon length. (**b**) RT–PCR analysis showing the expression pattern of CD44 variants in pGIPZ PC3 cells obtained using primers described in (**a**). (**c**, **d**) Quantitative RT–PCR analysis showing the expression levels of CD44 variants in pGIPZ- (**c**) and pLKO (**d**)-transfected PC3 cells. Data are presented as the enrichment of variable exons versus a constant region (spanning between the constitutive CD44 exon 6 and exon 7) set to 1 in cells transfected with the control plasmid (scr). Total levels of CD44 mRNA (bar graph on the left side of each image) were obtained by normalizing values obtained for the CD44 constant region to those of GAPDH. Values are means ± s.d. of three independent analyses. $*P < 0.05$.

were more affected by SAM68 expression than the downstream variable exons. Hence, these results indicate that SND1 and SAM68 are both required for the regulation of the endogenous *CD44* pre-mRNA AS in PC3 cells.

SND1 does not modulate the RNAPII elongation rate in CD44

Transcriptional regulators can influence splicing decisions through the modulation of the transcription elongation rate.[8,10] For instance, the SWI/SNF subunit BRM, which also interacts with SAM68, was suggested to slow down RNAPII in the region encoding the variable exons of *CD44*, thereby favoring their recognition by the spliceosome and their inclusion in the mRNA.[50] To test whether SND1 also had its role in splicing by modulating the RNAPII elongation rate within the *CD44* transcription unit, we analyzed the accumulation of nascent *CD44* pre-mRNA following reversible inhibition of transcription by 5,6-Dichlorobenzimidazole 1-β–D-ribofuranoside (DRB) treatment.[51] Control (scrambled si-RNA; si-scr) and SND1-depleted (si-SND1) PC3 cells were harvested at different time points from the removal of the drug, and pre-mRNA expression was detected by performing qPCR using primers that span the exon–intron junction. Contrary to what observed with BRM, we found that knockdown of SND1 has no significant effect on the RNAPII transcription rate, neither in the proximal part of the *CD44* gene nor in the region containing the variable exons (Supplementary Figure S5B). Thus, SND1 does not affect *CD44* AS by modulating the elongation rate of RNAPII.

SND1 affects the recruitment of SAM68 and snRNPs on CD44 pre-mRNA

SND1 interacts with the snRNPs and accelerates the kinetics of spliceosome assembly, thereby favoring constitutive pre-mRNA splicing.[36,37] We hypothesized that SND1 may exert its stimulatory effect on *CD44* AS by promoting efficient assembly of the spliceosome within the variable region of the gene. To test this possibility, we labeled nascent pre-mRNAs by incubating the cells for 45 min with BrU after their release from the DRB block. The ratio between *CD44* mRNA and pre-mRNA was then measured in the BrU-labeled nascent transcripts that were purified by immunoprecipitation. Knockdown of SND1 strongly impaired splicing of exon v5, whereas it had no significant effect on the efficiency of splicing of constitutive exons located upstream of the variable region (Figure 5a). The reduced splicing efficiency in the nascent transcript was still observed near a downstream variable exon (v9); however, it was notably attenuated in the constitutive region at the 3′ end of the gene (Figure 5a).

SND1 was shown to promote constitutive splicing by recruiting the snRNPs through its Tudor domain.[36] Strikingly, we found that deletion of this domain strongly comprised the ability of SND1 to enhance the inclusion of exon v5 (Figure 5b). Recruitment of the U5 snRNP by the spliceosome is required for its catalytic activity.[1] RNA immunoprecipitation experiments using an anti-PRP6 antibody revealed that knockdown of SND1 correlated with a decreased recruitment of the U5 snRNP on the *CD44* pre-mRNA in PC3 cells (Figure 5c), indicating that SND1 favors assembly of the active spliceosome. Moreover, SAM68 was also shown to associate with the U5 snRNP under conditions in which exon v5 inclusion was induced,[50] and we found that knockdown of SND1 strongly reduced the association of SAM68 with the *CD44* pre-mRNA (Figure 5d). Notably, we also found that the interaction of SAM68 with RNAPII[52] was partially compromised in SND1-depleted PC3 cells (Figure 5e). Altogether, these findings strongly suggest that SND1 acts as a bridge between RNAPII and SAM68 and has a key role in *CD44* AS by favoring the recruitment of the spliceosome and the efficient splicing of the variant exons.

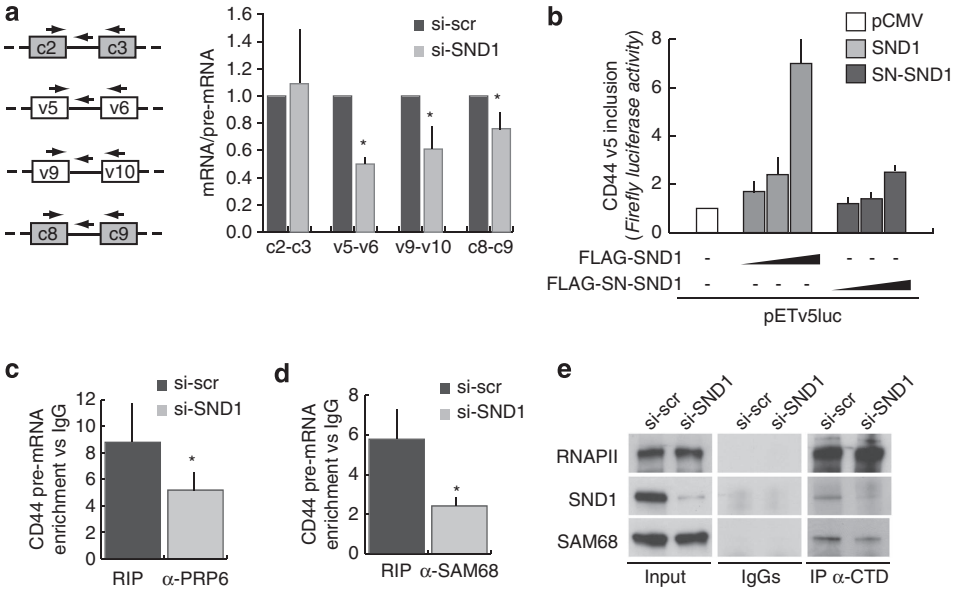

Figure 5. SND1 recruits SAM68 and U5 snRNP components to the CD44 pre-mRNA. (**a**) qPCR analysis of nascent CD44 transcripts labeled in the presence of BrU. Novel transcripts were immunoprecipitated by using specific anti-BrU antibody and analyzed with the indicated primers (left side). Cells not treated with BrU were used as control of the immunoprecipitation. The graph represents the ratio between mRNA versus pre-mRNA of specific CD44 regions. The value of si-scr-infected cells was set as reference. (**b**) Luciferase assay in HEK293T cells transfected with pETv5luc construct in presence of increasing amount of wild-type FLAG-SND1 (light gray bars) or FLAG-SN-SND1 (dark gray bars), Renilla luciferase plasmid (used as internal transfection control). Bar graph indicates the normalized luciferase activity ± s.d. from three independent transfections. (**c**, **d**) RNA immunoprecipitation assays performed in pGIPZ PC3 clones to detect the recruitment of PRP6 (**c**) or SAM68 (**d**) on the endogenous CD44 pre-mRNA. The immunoprecipitation was performed using the indicated antibodies or control IgGs. The graph represents the enrichment of CD44 pre-mRNA bound to PRP6 or SAM68 relative to IgGs. The qPCR analysis was performed using exon–intron spanning primers surrounding the variable exon v5 (mean ± s.d. of three independent experiments). (**e**) Western blot analysis of the co-immunoprecipitation between the endogenous SAM68 and RNAPII performed in SND1-depleted PC3 cells. Nuclear extracts were immunoprecipitated with nonspecific IgGs and anti-RNAPII antibody. $^*P < 0.05$.

Knockdown of SND1 and SAM68 inhibits PC3-cell migration

The inclusion of variable exons in *CD44* promotes cell proliferation and motility in human cancer cells.[47,49,53] Previous data indicated that silencing of SND1 by small interfering RNAs (siRNAs) causes a significant decrease in PC3 cell growth.[43] As SAM68 was also shown to exert the same effect in the less-aggressive LNCaP cell line,[29] we first asked whether SND1 and SAM68 cooperate in supporting PCa cell proliferation. Transient knockdown of SND1 and SAM68 by RNAi in PC3 cells efficiently reduced the expression levels of both proteins at 48 h after transfection (Supplementary Figure S6A). Downregulation of either SND1 or SAM68 alone caused a small negative effect on the growth of PC3 cells (Supplementary Figure S6B). However, proliferation was significantly reduced in cells that were concomitantly depleted for both proteins, suggesting that high levels of SAM68 and SND1 are required for optimal proliferation of the metastatic PC3 cell line.

Knockdown of CD44v5 reduced migration of cancer cells in culture.[47] Similarly, we found that transient silencing of either SND1 or SAM68 reduced PC3 cell migration in a trans-well migration assay (Supplementary Figure S7). Importantly, the same effect was also observed by stable knockdown of these proteins obtained with a different siRNA (Figures 6a and b). Thus, our findings indicate that increased expression of SAM68 and SND1 may provide an advantage to PCa cells to migrate and invade other tissues.

DISCUSSION

The study presented here identifies SND1 as a novel SAM68-interacting protein in PCa cells. We provide evidence that SND1 forms a complex with SAM68 and positively regulates its splicing activity. In particular, we report that SND1 enhances SAM68-mediated splicing of specific variable exons in *CD44* mRNA—an event associated with tumor progression and metastasis.[49]

SAM68 was previously shown to favor *CD44* splicing upon MAPK activation triggered by phorbol esters.[22] This stimulation also enhanced the interaction of SAM68 with the U5 snRNP and the recruitment of the chromatin-remodeling protein BRM. Assembly of this complex within the *CD44* transcription unit was proposed to slow down the RNAPII and to allow the inclusion of the *CD44* alternative exons.[50] Our experiments suggest that SND1 affects *CD44* AS in a slightly different manner. Indeed, we found that, although SND1 binds to the RNAPII, it does not affect its local transcription elongation rate within the *CD44* gene. On the other hand, we show that expression of SND1 is required for the efficient association of SAM68 with RNAPII and for the recruitment of SAM68 and PRP6, a U5 snRNP protein component, on the *CD44* pre-mRNA. These effects of SND1 correlate with a more efficient splicing of the variable exons in the nascent *CD44* pre-mRNA. Hence, we propose a model in which SND1 travels with the RNAPII along the *CD44* transcription unit and acts as a scaffold protein to recruit the factors required for efficient splicing of the gene (Figure 7). In line with our hypothesis, SND1 was reported to accelerate the kinetics of spliceosome assembly by directly interacting with the U5 snRNP.[36] In this scenario, SND1 would coordinate pre-mRNA processing via interaction with snRNPs and splicing regulators, similar to SAM68, whose binding to the nascent *CD44* transcript is perturbed upon SND1 knockdown. Accordingly, we found that an SND1 mutant deficient in recruitment of the snRNPs did not enhance exon v5 inclusion. Consistent with our model is also the observation that SAM68 is required for the effect of SND1 on *CD44* splicing, as demonstrated by knockdown experiments and by introducing mutations of the

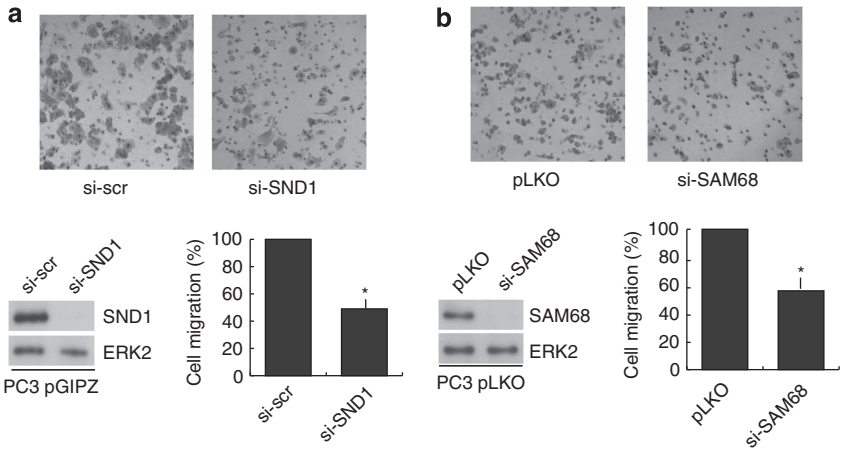

Figure 6. Knockdown of SND1 impairs migration of PC3 cells. Transwell migration assay of PC3 clones stably knocked down for SND1 (**a**) or SAM68 (**b**). Cells were resuspended in serum-free medium and placed in a transwell chambers and induced to migrate toward serum-containing medium for 16 h. Representative images are shown on the top of each panel. The percentage of the migrated cells is indicated at the bottom of the photographs together with the immunoblot analysis showing the expression levels of SND1, SAM68 or ERK2 used as loading control. The percentage of cell migration was normalized to that of control cells. $*P < 0.05$.

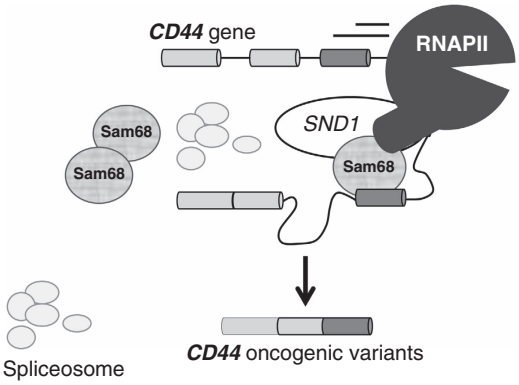

Figure 7. Schematic model for the regulation of CD44 alternative splicing by SND1 and SAM68. SND1 acts as a scaffold protein to bridge the RNAPII with spliceosomal components and the splicing regulator SAM68 during transcription of the CD44 pre-mRNA. The presence of SAM68 allows the recognition of weak splice sites in the variant exons from the spliceosomal apparatus, promoting their inclusion. The high levels of expression of SND1 and SAM68 in PCa cells might favor AS events that results in the production of oncogenic CD44 variants involved in invasiveness and cancer progression.

SAM68-binding sites in the *CD44* pre-mRNA. Furthermore, we found that SND1 did not directly bind to CD44 pre-mRNA, neither *in vitro* nor *in vivo* (data not shown). Thus, we speculate that the recruitment of specific splicing factors, similar to SAM68, is required to direct SND1 activity towards weak exons in selected pre-mRNAs, thereby favoring the local assembly of the spliceosome. With regard to our study, SND1 may modulate *CD44* AS by reinforcing the interaction of SAM68 with the RNAPII and by favoring its binding to the nascent *CD44* pre-mRNA. In turn, SAM68 may signal to the spliceosome the presence of weak splice sites of the *CD44* variable exons, thus promoting their inclusion. This mechanism may not be necessary, or less relevant, for constitutive exons, which are normally recognized with high efficiency by the splicing machinery.

Although several observations link SND1 to carcinogenesis, little is known about the role of this protein in cancer cells. An increased SND1 activity in the RNA-induced silencing complex was found to correlate with the degradation of mRNAs encoding tumor-suppressor proteins by oncogenic miRNAs, thereby promoting the development of hepatocellular carcinoma.[42] Moreover, it was reported that SND1 contributes to tumor angiogenesis by favoring the transcriptional activity of NF-kB, which in turn allows the expression of genes involved in neovascularization.[54] Our results now suggest that the regulation of AS by SND1 may also contribute to malignant transformation possibly by regulating cancer-relevant genes such as *CD44*. Interestingly, a role in AS has been recently shown also for the Argonaute (AGO) proteins, which are the core components of the RNA-induced silencing complex. In particular, it was described that AGO proteins interact with splicing factors and with the U5 snRNP. This interaction would then favor the inclusion of *CD44* variable exons by decreasing the elongation rate of the RNAPII.[55] As SND1 interacts with AGO2 in the RNA-induced silencing complex, and it directly binds to the U5 snRNP, it is possible that the SND1-containing complex in the *CD44* gene is larger than that represented in our model and it also includes the AGO proteins. In this regard, it is noteworthy that SAM68 was also recently shown to interact with proteins involved in miRNA biogenesis and to regulate the expression of selected miRNAs.[56]

Misregulation of cancer-associated AS events is often correlated to unbalanced expression of splicing factors. We detected an increased expression of both SND1 and SAM68 in PCa cells with respect to a benign prostate cell line. A previous proteomic profile identified high SND1 expression in metastatic breast cancer cells and in tumor samples of metastatic breast cancer patients.[39] Moreover, microarray analyses indicated that depletion of SND1 in breast cancer cells leads to the down\regulation of genes associated with metastasis and chemoresistance.[40] SAM68 is also upregulated in breast cancer cells, and its expression correlates with malignant and aggressive phenotypes.[57] Given their concomitant upregulation in breast cancer cells, it would be interesting to determine whether or not splicing of SND1-regulated genes is also altered and whether SAM68 is required for this regulation.

In conclusion, we provide evidence that SAM68 and SND1 act in complex in the regulation of AS of the variable exons of *CD44*, which are known to promote cell motility. Consistently, we document that the knockdown of either protein strongly reduced the migratory ability of PC3 cells. Hence, the SND1/SAM68 complex may be an important determinant of PCa progression and the concomitant upregulation of these proteins may provide an advantage to cancer cells to invade other tissues, thus favoring the metastatic process.

MATERIALS AND METHODS

Cell culture and transfections

LNCaP cells were cultured in RPMI 1640 medium (Lonza, Walkersville, MD, USA), HEK293T and PC3 cells in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO, USA), all supplemented with 10% fetal bovine serum (FBS; Lonza), antibiotics. For transfections, cells were plated in 35-mm dishes and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with 1 μg of DNA: CD44 pETv5 minigene,[22] pEGFP-SAM68, pCMV5FLAG-SND1, pCMV5FLAG-SN-SND1 and pCDNA3myc-SAM68. After 24 h, cells were collected for protein and RNA analyses. For RNA interference, cells at ~60–70% confluence were transfected with siRNAs (Sigma-Aldrich) using Lipofectamine RNAiMAX (Invitrogen). Sequences for SAM68 siRNA were previously described.[29] The SND1 siRNA used was: 5′-UCUUUCUUCUGCUUUGCGG-3′. Scrambled siRNA was: 5′-GUGCUCAA UUGGAUUCUCU-3′.

Stable knockdown of SAM68 and SND1 in PC3 cells

PC3 cells were transfected with the lentiviral construct pGIPZ containing scrambled sequence siRNA (pGIPZscr) or SND1 siRNA (pGIPZsiSND1) (Openbiosystem, Huntsville, AL, USA). Puromycin (0.5 μg/ml) (Sigma-Aldrich) was added to fresh medium every 3 days and resistant colonies were selected after 10 days, picked and expanded.

To obtain pLKO clones, PC3 cells were infected by adding either control pLKO or pLKO si-SAM68 lentiviral particles to serum-free medium supplemented with polybrene (4 μg/ml) as previously described.[58] Stable clones expressing siRNAs were expanded and maintained by adding 0.5 μg/ml Puromycin.

RT–PCR and real time PCR analyses

Cellular RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instructions and subjected to DNase (Roche, Indianapolis, IN, USA) digestion. One microgram of total RNA was used for RT–PCR using M-MLV reverse transcriptase (Invitrogen). Five percent of the RT reaction was used as template for PCR analysis (GoTaq, Promega, Madison, WI, USA). Quantitative real-time PCR was performed using the SYBR Green Master Mix (for Light-Cycler 480; Roche) according to the manufacturer's instructions. Primer sequences are listed in the Supplementary Table 2. Statistical analysis was performed using Student's *t*-test.

Protein extraction and western blot analysis

Cell extracts, cytosol/nuclear fractionations,[23,58] sucrose gradient fractionation of the nuclear compartment[59] and western blot analysis[23] were performed as previously described. Primary antibodies used (1:1000 dilution; overnight at 4 °C) were the following: rabbit anti-ERK2, mouse anti-Myc, rabbit anti-SAM68 and goat anti-lamin B (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-tubulin, mouse anti-FLAG (Sigma-Aldrich) and rabbit anti-GFP (Molecular Probes, Eugene, OR, USA), mouse anti-RNAPII (CTD4H8; Millipore, Billerica, MA, USA) and mouse anti-SND1.[39]

Immunoprecipitation experiments

Nuclear extracts were used in co-immunoprecipitation experiments as previously described[23,58] using 2 μg of anti-SND1, anti-FLAG, anti-RNAPII or anti-SAM68 antibodies and either mouse or rabbit IgGs as control. After three washes with lysis buffer, proteins were eluted in sodium dodecyl sulphate sample buffer for western blot analysis.

GST pull-down experiments

GST fusion proteins were expressed in *Escherichia coli* BL21 strain and purified from the bacterial lysate as previously described.[60] Upon binding to glutathione-agarose beads (Sigma-Aldrich), recombinant proteins were incubated with LNCaP cell extracts for 2 h under constant rotation. Beads were washed three times with lysis buffer and bound complexes were eluted in sodium dodecyl sulphate sample buffer for western blot analysis.

Luciferase reporter assay

HEK293T cells (1×10^5) were cultured in 12-well plates and transfected with the indicated constructs together with 90 ng of wt or mutated CD44 pETv5 luciferase[48] and the *Renilla* luciferase reporter gene (1 ng) as internal control. Twenty-four hours after transfection, cells were harvested, lysed and analyzed with a biocounter luminometer using the dual-luciferase reporter assay system (Promega). Data were normalized for transfection efficiency using the ratio between firefly and Renilla luciferase activity.

MTS Proliferation assay and Migration assay

Cell proliferation was determined using the CellTiter A96 MTS method according to the manufacturer's instructions (Promega) by plating 1.2×10^3cells/well in 96-well culture plates. Migration assay was performed using migration chambers (Becton Dickinson, San Jose, CA, USA) and FBS as chemoattractant as previously described.[47]

Chromatin immunoprecipitation and RNA immunoprecipitation

PC3 cells were plated in 150-mm dishes (5×10^6) 1 day before and treated with 75 μM DRB (Sigma) for 6 h to inhibit transcription and synchronize them. Cells were then washed twice with phosphate-buffered saline to remove the DRB and incubated with fresh medium for 45 min. One percent (vol/vol) formaldehyde was added to the medium at the end of incubation for 10 min at room temperature, and cells were lysed as described[24] and sonicated with Bioruptor (Dyagenode) to yield chromatin size of ~300 base pairs. Seventy microgram of DNA/sample were used for immunoprecipitation with 3 μg of anti-SND1 antibody or control mouse IgGs. Input DNA was collected from the supernatant of samples immunoprecipitated with no antibody. Immunoprecipitated DNA was analyzed by qPCR.

For RNA immunoprecipitation experiments, PC3 pGIPZ clones treated with DRB were crosslinked with 1% (vol/vol) formaldehyde for 10 min at room temperature and processed as described.[61] To label nascent pre-mRNAs, after the removal of DRB, 2 mM of BrU was added to the fresh medium for 45 min. Labeled pre-mRNA was immunoprecipitated with 1 μg of antiBrdU antibody (Becton Dickinson) and isolated as described.[62]

CONFLICT OF INTEREST

The authors declare no conflict of interests.

ACKNOWLEDGEMENTS

We wish to thank Dr Roberta Busà for suggestions with chromatin immunoprecipitation experiments and Dr Maria Loiarro for assistance with luciferase reporter assays. This work was supported by the Association for International Cancer Research (AICR grant no. 12-0150 to CS) and the Associazione Italiana Ricerca sul Cancro (AIRC grant no. 10348 to CS and MFAG no. 11658 to MPP).

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