

Protein Sam68 regulates the alternative splicing of survivin DEx3

Received for publication, June 2, 2017. Published, Papers in Press, June 27, 2017. DOI 10.1074/jbc.M117.800318

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Edited by Eric R. Fearon

Messenger RNA alternative splicing (AS) regulates the expression of a variety of genes involved in both physiological and pathological processes. AS of the anti-apoptotic and proliferation-associated survivin (*BIRC5*) gene generates six isoforms, which regulate key aspects of cancer initiation and progression. One of the isoforms is *survivin* DEx3, in which the exclusion of exon 3 generates a unique carboxyl terminus with specific anti-apoptotic functions. This isoform is highly expressed in advanced stages of breast and cervical tumors. Therefore, understanding the mechanisms that regulate *survivin* DEx3 mRNA AS is clearly important. To this end, we designed a minigene (M), and in combination with a series of deletions and site-directed mutations, we determined that the first 22 bp of exon 3 contain *cis*-acting elements that enhance the exclusion of exon 3 to generate the *survivin* DEx3 mRNA isoform. Furthermore, using pulldown assays, we discovered that Sam68 is a possible *trans*-acting factor that binds to this region and regulates exon 3 splicing. This result was corroborated using a cell line in which the Sam68 binding site in the *survivin* gene was mutated with the CRISPR/Cas system. This work provides the first clues regarding the regulation of *survivin* DEx3 mRNA splicing.

Alternative splicing (AS)⁴ is a fundamental mechanism for differential gene expression that generates distinct mRNAs from the same pre-mRNA sequence, yielding different protein isoforms with different cellular functions (1). Approximately 95% of all human genes are regulated by this mechanism (2). AS depends on the recognition of *cis*-acting splicing elements known as exonic splicing enhancers and silencers or intronic splicing enhancers and silencers (3). Serine/arginine-rich proteins (SR) and heterogeneous nuclear

ribonucleoproteins (hnRNPs) are two well-characterized types of *trans*-acting splicing factors that interact with exonic and intronic enhancers or silencers to assist the spliceosome in exon recognition (4, 5).

The importance of accurate AS is demonstrated by the fact that alterations in *cis*-acting elements and the deregulation of *trans*-acting factors are involved in several human diseases (6). For example, ~15 to 50% of human disease mutations affect splice site selection; therefore, elucidating the AS code is clearly important (7, 8). In cancer, many reports have demonstrated a relationship between disease progression and deregulated AS for several genes implicated in apoptosis, cell cycle control, invasion, metastasis, and angiogenesis (9). One example is the *BIRC5* gene, which encodes survivin, a protein involved in inhibiting apoptosis and regulating the cell cycle (10). AS of the *BIRC5* gene produces six isoforms with similar or antagonistic functions as follows: anti-apoptotic survivin; anti-apoptotic *survivin* DEx3, which lacks exon 3; *survivin* 2B+32, which is subjected to nonsense-mediated decay; pro-apoptotic *survivin* 2B, which retains part of intron 2; anti-apoptotic *survivin* 3B, which retains part of intron 3; and pro-apoptotic *survivin* 2 α , which arises from the loss of exons 3 and 4 and the insertion of the 3' UTR, which generates a premature stop codon (11). However, the mechanism by which splice variants of *survivin* regulate apoptosis is not fully understood.

One of the most important characteristics of *survivin* and its splice variants is that they are almost universally up-regulated in cancer, whereas they are scarcely detectable in normal cells (12). In addition, overexpression of *survivin* and its isoforms correlates with cancer progression and treatment outcomes in many types of cancer (13). Interestingly, whereas *survivin* shows constant high expression in diverse types and stages of cancer, the expression of *survivin* isoforms is variable and depends on the specific type and stage of cancer, pointing to a cancer-associated AS regulation mechanism (10, 14). Because of these expression differences, some isoforms are considered to be better prognostic or diagnostic markers than *survivin* itself. For example, *survivin* DEx3 shows differential overexpression compared with other *survivin* variants in breast cancer, bladder cancer, and sarcomas. In addition, *survivin* DEx3 has been proposed as a useful diagnostic marker in breast cancer, papillary thyroid carcinoma, and lung cancer (15–19). The overexpression of this isoform is also associated with poor

This work was supported by Consejo Nacional de Ciencia y Tecnología Grant 132931. The authors declare that they have no conflicts of interest with the contents of this article.

This article contains supplemental Figs. S1–S3 and supplemental Table S1.

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⁴ The abbreviations used are: AS, alternative splicing; M, minigene; hnRNP, heterogeneous nuclear ribonucleoprotein; dNTP, deoxyribonucleotide triphosphate; qPCR, quantitative PCR; dpi, days post injection.

Survivin DEx3 alternative splicing regulation

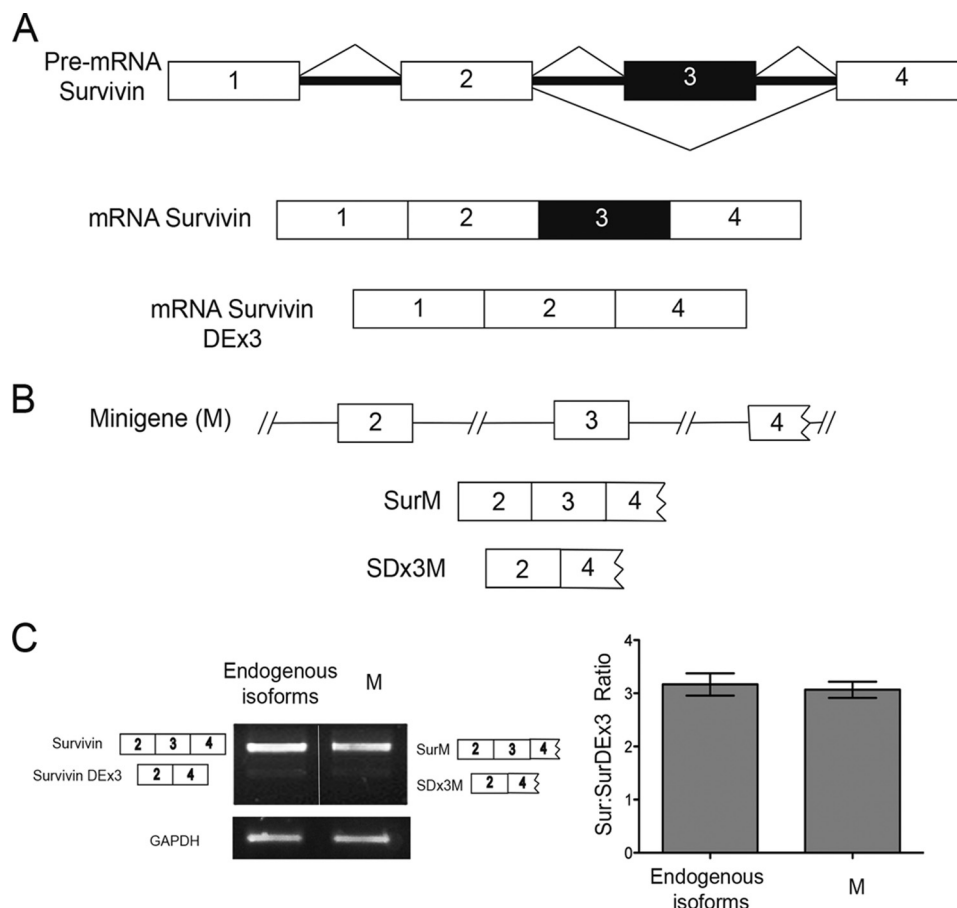


Figure 1. Expression of the minigene in HeLa cells. *A*, structure of the survivin pre-mRNA and spliced mRNA isoforms. Exons 2 and 4 are represented by white boxes, exon 3 by a black box, and introns with black lines. *B*, schematic representation of the minigene and the two isoforms generated through alternative splicing: SurM, which includes exon 3, and SDx3M, which excludes exon 3. Both isoforms include exons 2 and 4. *C*, RT-PCR analysis of the expression of the minigene (M) and endogenously spliced mRNA isoforms using specific primers for each case. GAPDH was used as a constitutive gene. Bar graph showing the relative expression of endogenous and M isoforms (mean ± S.D.; $n = 3$).

responses to chemotherapy and low patient survival in cases of soft tissue sarcoma, glioma, astrocytoma, and gastric cancer (16, 20, 21). The differences in the cellular effects of survivin DEx3 and survivin have been attributed to their alternative C termini. In addition to its apoptosis-regulating activity, the survivin DEx3 carboxy-terminal region has domains that are not present in other isoforms, including a mitochondrial localization domain, a nuclear localization domain, and a BH2 domain. This last domain is characteristic of another protein family involved in apoptosis regulation, the Bcl-2 family (22). The association of survivin DEx3 with Bcl-2 through the BH2 domain confers greater stability in the interaction of survivin DEx3 with caspase 3, leading to inhibition of apoptosis (22). To date, no reports have explored the *cis*-acting elements or *trans*-acting factors that regulate survivin DEx3 splicing. Because of the relevance of this isoform in cancer progression, studying the mechanisms through which survivin DEx3 is generated not only is relevant for advancing our understanding of cancer but will also potentially contribute to the development of new markers or therapeutic alternatives. In the present study, we evaluated the role of *cis* elements and *trans* factors that control the exclusion of survivin exon 3 using a minigene (M) construct

model. In addition, we identified Sam68 as a *trans*-acting factor that regulates this process.

Results

Minigene expression

The *survivin* gene contains 4 exons and 3 introns, which generate six isoforms, including survivin DEx3, which lacks exon 3 (Fig. 1*A*). To determine the elements that regulate exon 3 skipping, we designed an M that contains the necessary elements to carry out splicing of this exon (Fig. 1*B*). Because several reports (23) suggest that most of the splicing regulatory elements are contained within the 300 bp that surround an exon, we focused on the 700 bp upstream and downstream of exon 3. The survivin M was expressed in HeLa cells under the control of the minimal cytomegalovirus promoter. Two transcripts were expressed from the M as follows: SurM, which contains exons 2, 3, and 4, and SDx3M, which contains only exons 2 and 4 (Fig. 1*B*). Similar to endogenous survivin mRNA, the expression of the SurM isoform was predominant over that of the SDx3M isoform, at a 3:1 ratio (SurM:SDx3M) (Fig. 1*C*). We concluded that the M undergoes the same process as the endogenous isoforms. Therefore, our model can be used to identify *trans*- and

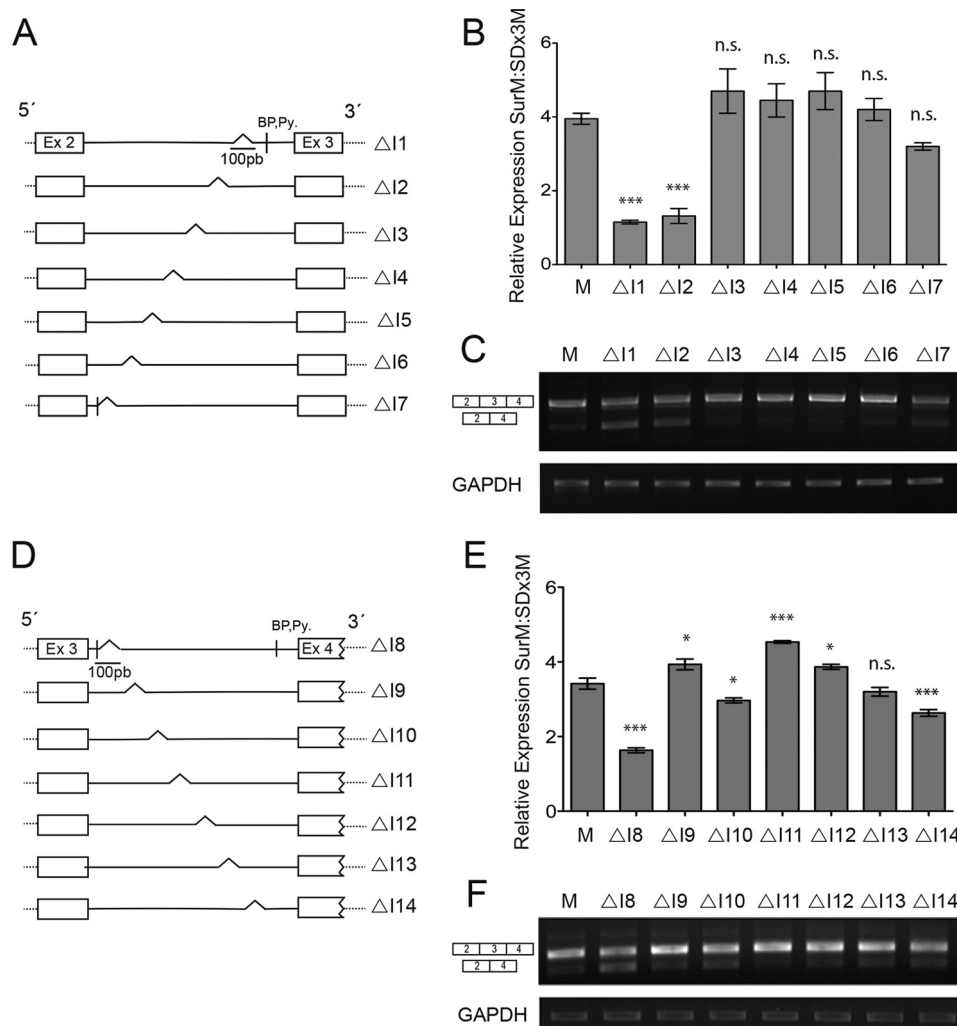


Figure 2. Effect of intron deletions on the inclusion or exclusion of exon 3 in a minigene assay is shown. A–D, schematic diagram of the minigene (M) intronic deletions of 100 bp in each intron. Exons are represented by white boxes and introns by black lines; consensus regions such as branch points (BP) and polypyrimidine tracts (Py) are indicated. B, bar graphs showing the ratio of SurM and SDx3M isoform expression (mean $\pm$ S.D.; $n = 3$) as affected by each deletion introduced into the intron upstream of exon 3. C, representative RT-PCR analyses of HeLa cells transfected with constructs containing the Δ I1 to Δ I7 deletions. E, bar graph showing the relative expression of SurM:SDx3M (mean $\pm$ S.D.; $n = 3$) affected by each deletion introduced into the intron downstream of exon 3. F, representative RT-PCR analysis of HeLa cells transfected with Δ I8 to Δ I14. GAPDH was used as a constitutive gene. The p values from one-way ANOVA are reported. *, $p < 0.05$; ***, $p < 0.001$; n.s., not significant.

cis-regulatory elements that are necessary to modulate exon 3 exclusion.

Identification of intronic and exonic *cis* elements that regulate survivin AS

To identify intronic *cis*-acting elements, we designed 14 deletions in the two introns surrounding exon 3, including seven 100-bp deletions per intron (Fig. 2, A and D). To avoid deleting the regulatory splicing consensus sequences, 60 bp were left in the 5' region, and 10 bp were left in the 3' region of the exon 3 (24). Constructs containing all of these deletions were transfected into HeLa cells, followed by RT-PCR analyses. Even when SurM continued to be the main isoform expressed, we observed that each deletion had a different effect on exon 3 exclusion, as reflected by the ratio of the two isoforms. Specifically, the Δ I1 and Δ I2 deletions, located upstream of exon 3, had a significant effect on the expression ratio of the two isoforms (Fig. 2B), with an increase in SDx3M isoform expression

(exon 3 exclusion) and a slight decrease in the SurM isoform (Fig. 2C) compared with the M. Therefore, these deletions affect the splicing of the SDx3M isoform and to a lesser extent the SurM isoform. We also found that the Δ I3, Δ I4, Δ I5, and Δ I6 deletions tended to favor the SurM isoform, although no significant differences were observed. In the case of deletions downstream of exon 3 (Fig. 2D), removing the first 100 bp (deletion Δ I8) (Fig. 2, E and F) induced an increase in the expression of the SDx3M isoform (an increase in the exclusion of exon 3). The Δ I9, Δ I10, Δ I11, Δ I12, and Δ I14 deletions (Fig. 2E) also resulted in significant differences in the ratio of the two M isoforms, although not to the extent of Δ I8. Hence, these results suggest that the 200 bp upstream and 100 bp downstream of exon 3 contain important elements that control the exclusion/inclusion of exon 3 and therefore modulate splicing of the SDx3M isoform.

We next sought to identify the most relevant exonic *cis*-acting elements. For this purpose, we designed three deletions in exon 3. The first deletion, Ex3 Δ 1, lacks 22 bp; the second dele-

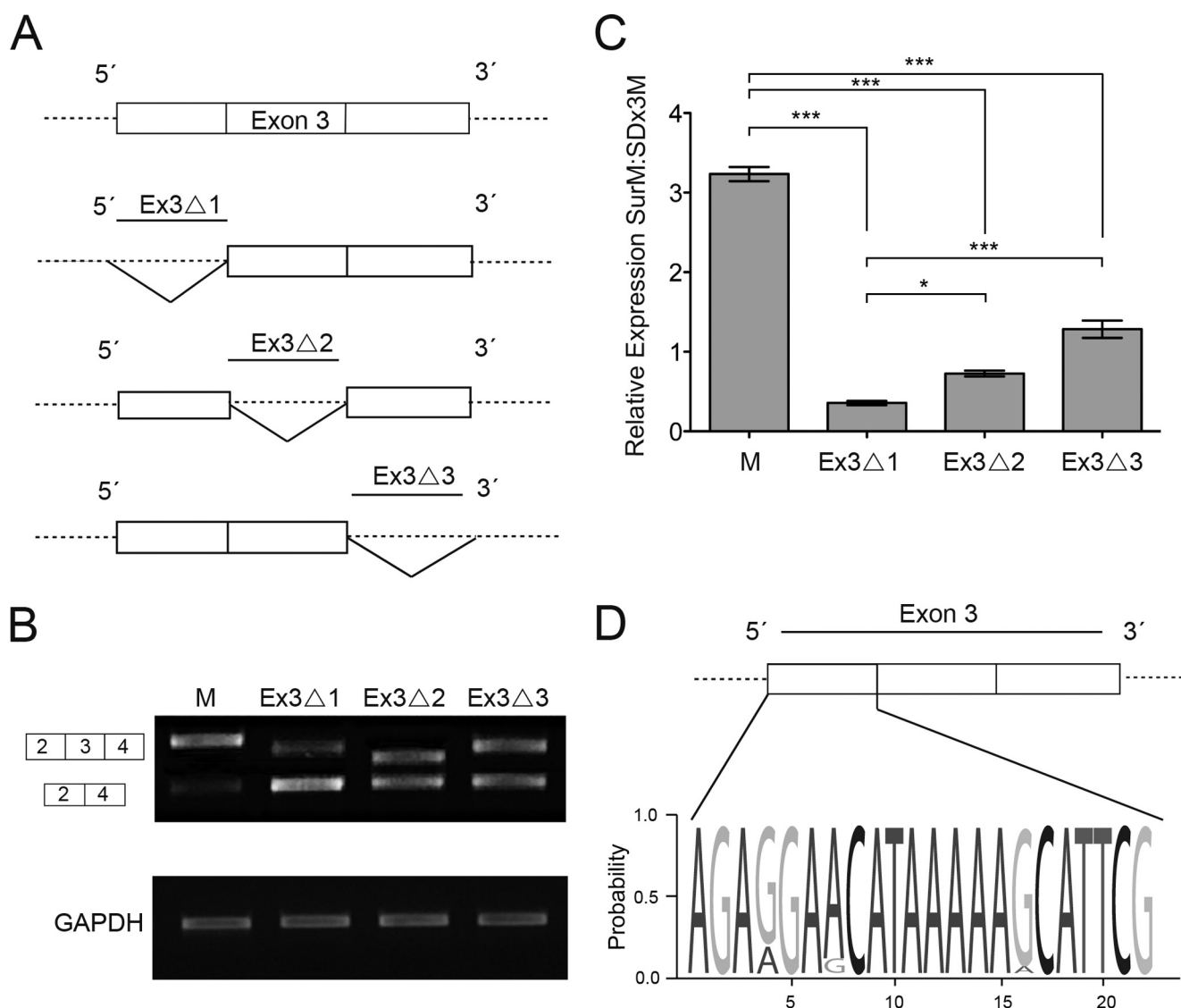


Figure 3. Effect of exon deletions on the inclusion or exclusion of exon 3 in a minigene assay is shown. A, schematic diagram of the minigene (M) deletions in exon 3. Exons are represented by white boxes, and the positions of the deletions are indicated with an underlined Ex3Δ# (# indicates the number of the deletion). B and C, the splicing patterns were analyzed via RT-PCR of HeLa cells transfected with constructs containing the deletions indicated in A: M (control), Ex3Δ1, Ex3Δ2, and Ex3Δ3. The bar graph shows the ratio of SurM:SDx3M expression (mean $\pm$ S.D.; $n = 3$) (upper panel). D, sequence alignment of the first 22 bp of exon 3 from 15 different species with WebLogo software (47). GAPDH was used as a constitutive gene. The p values from one-way ANOVA are reported. *, $p < 0.05$; ***, $p < 0.001$; n.s., not significant.

tion, Ex3Δ2, lacks 49 bp; and the third deletion, Ex3Δ3, lacks 37 bp (Fig. 3A). Minigenes containing the three deletions were transfected into HeLa cells followed by RT-PCR analyses. We observed that each deletion induced an increase in the SDx3M isoform with respect to the M-transfected cells. The Ex3Δ1 deletion resulted in the greatest change in expression regarding exon 3 exclusion, shifting the isoform ratio from 3:1 SurM:SDx3M in M to 1:10. Both the Ex3Δ2 and the Ex3Δ3 deletions induced a significant increase in the SDx3M isoform, shifting the ratio from 3:1 to 1:1.3 and 1:1, respectively (Fig. 3, B and C). These changes suggested that the most important splicing regulatory elements are present within the first 22 bp of the exon, although we do not exclude the possibility that elements present in the other two deletions examined also play a role. Accordingly, alignment of the 22-bp region among 15 different species revealed a high degree of conser-

vation (Fig. 3D). Because of these results, we focused on this 22-bp region.

Cis-exonic mutations affecting exon 3 exclusion

We next performed an *in silico* analysis of the 22-bp exonic region with three different tools that predict potential binding *trans* factors (Fig. 4A). These analyses revealed the presence of three regions, ExR1, ExR2, and ExR3. To assess the relevance of the putative binding sites, we generated constructs with point mutations in each region (Mut1Ex3, Mut2Ex3, and Mut3Ex3) (Fig. 4A). Minigenes containing these mutations were transfected into HeLa cells, and the effect of each construct was measured via RT-PCR. As shown in Fig. 4B, upper panel, the Mut1Ex3 and Mut3Ex3 constructs increased basal expression of the SDx3M isoform, whereas mutation of the ExR2 region (Mut2Ex3) dramatically increased the SurM isoform and

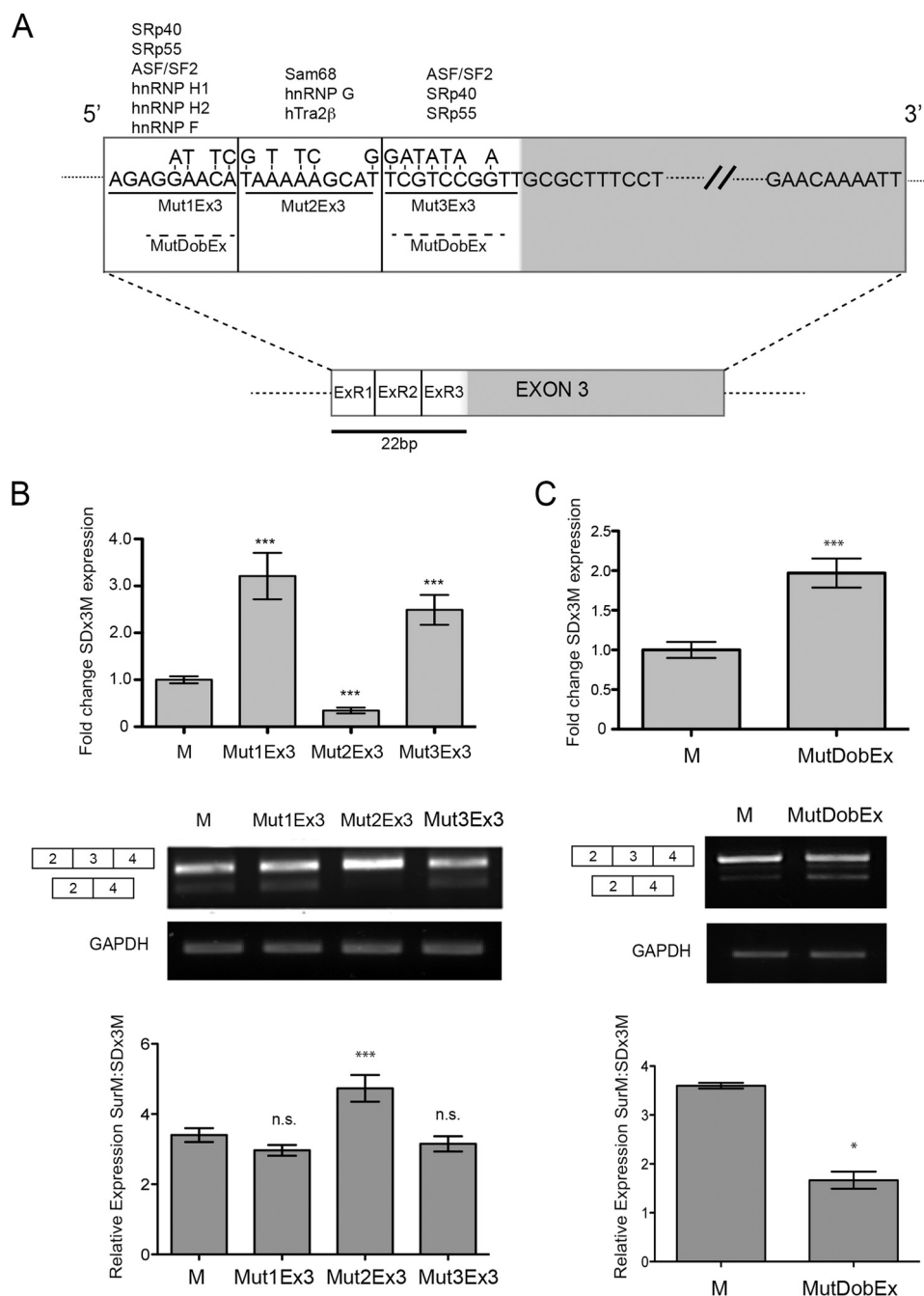


Figure 4. Mutations in the first 22 nucleotides of exon 3 alter the ratio of the SurM:SDx3M minigene isoforms. A, schematic diagram of exon 3 of the minigene (M), as well as the positions of the mutations in exon 3 and the positions of the putative *trans*-acting factors. B, bar graph (upper panel) showing the relative fold-change in SDx3M isoform mRNA expression determined via qPCR after M, Mut1Ex3, Mut2Ex3, and Mut3Ex3 were transfected into HeLa cells (mean $\pm$ S.D.; $n = 3$). RT-PCR analyses (middle panel) of HeLa cells transfected with M (control), Mut1Ex3, Mut2Ex3, and Mut3Ex3 showing the ratio of the two minigene isoforms. Bar graph (lower panel) showing the relative expression of SurM:SDx3M (mean $\pm$ S.D.; $n = 3$). C, bar graph (upper panel) showing the relative fold-change in SDx3M isoform mRNA expression determined via qPCR after M and MutDobEx were transfected into HeLa cells (mean $\pm$ S.D.; $n = 3$). RT-PCR analyses (middle panel) of HeLa cells transfected with M (control) and MutDobEx. Bar graph (lower panel) showing the relative expression of SurM:SDx3M (mean $\pm$ S.D.; $n = 3$). GAPDH was used as a constitutive gene for all assays. The p values from one-way ANOVA (B) and Student's t test (B and C) are reported. *, $p < 0.05$; ***, $p < 0.001$; n.s., not significant.

diminished the SDx3M isoform (thus increasing exon 3 inclusion). Nevertheless, only the Mut2Ex3 isoform ratio was significantly modified, as shown in Fig. 4B, lower panel. This result clearly contrasted with the effect observed for the Ex3Δ1 deletion, which corresponds to the complete 22-bp region, where we observed a 10-fold increase in the expression of the SDx3M isoform. This difference could be because of a possible inhibitory effect of the ExR1 and ExR3 regions on ExR2.

To test this hypothesis, we produced a double mutant (MutDobEx) of the ExR1 and ExR3 regions (Fig. 4A), which was expected to reverse the effect of the Mut2Ex3 construct (Fig. 4B) and increase the expression of the SDx3M isoform. Indeed, as shown in Fig. 4C, upper panel, we observed that, even when SurM remained as the main isoform expressed, MutDobEx increased the expression of the SDx3M isoform, enhancing the exclusion of exon 3. Therefore, MutDobEx mainly

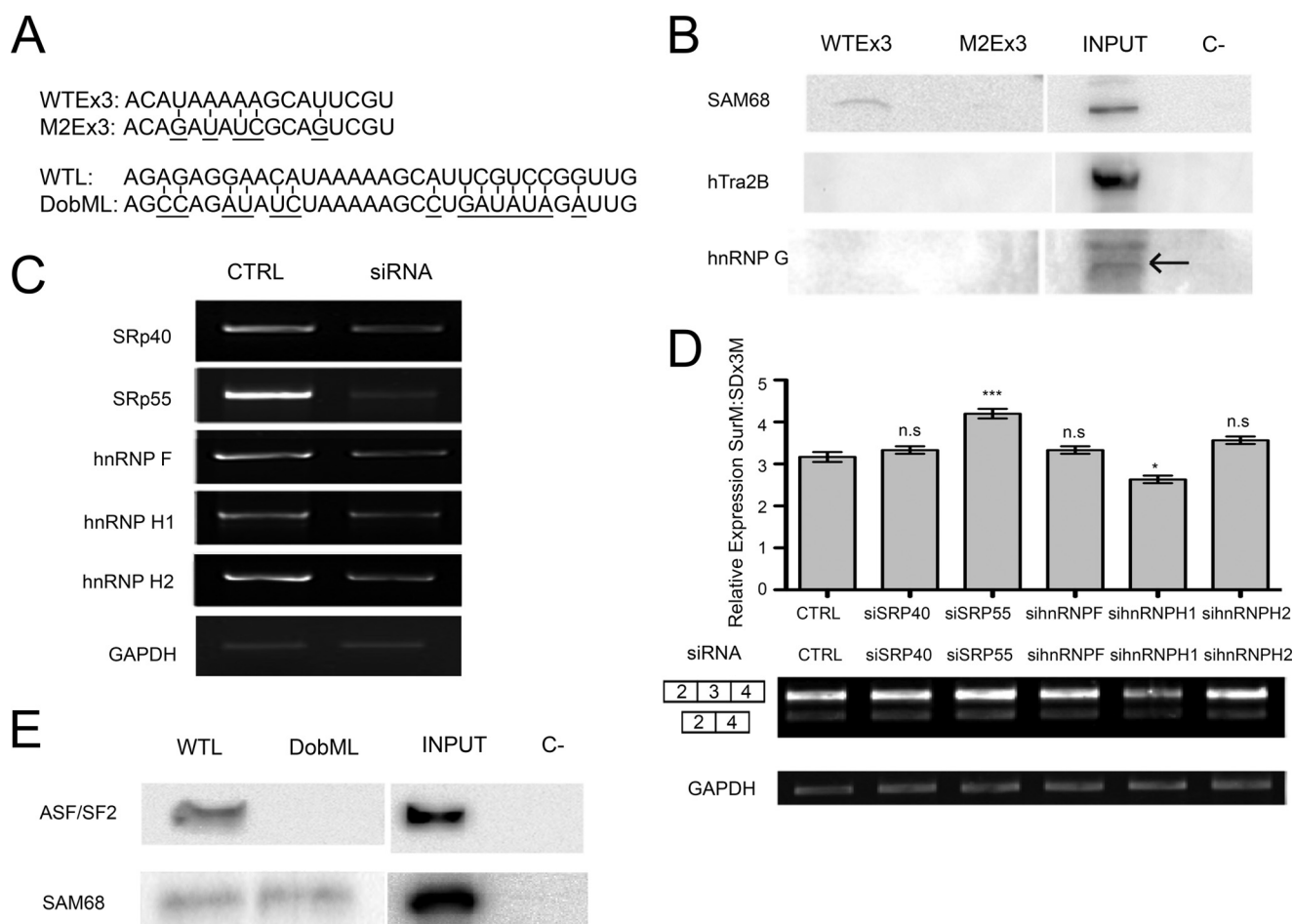


Figure 5. Sam68 binds to exon 3 of survivin. *A*, sequences of the RNA probes used for RNA pulldown assays. WTE_{x3}, wild-type sequence of the ExR2 region; WTL, wild-type sequence covering the first 22 bp of exon 3; M2Ex₃, mutation in the ExR2 region affecting the binding site of Sam68; DobML, mutation affecting the ExR1 and ExR3 regions. The positions of the mutated bases in the M2Ex₃ and DobML probes are underlined. *B*, Western blot analyses of pulldown assays using WTE_{x3} and M2Ex₃ biotin-labeled RNA probes; the probes were incubated with total HeLa protein; the input was total protein from HeLa cells (INPUT), and C-lane was the last wash. The pulled down proteins were detected using specific antibodies against Sam68, hnRNP G, and hTra2B. *C*, effect of the inhibiting *trans*-acting factors on the AS of the minigene. RT-PCR analysis of HeLa cells transfected with plasmids expressing shRNAs against *trans*-acting factors SRp40, SRp55, hnRNP F, hnRNP H1, and hnRNP H2. Equal amounts of cDNA were used for each amplification (as assessed by GAPDH RT-PCR). *D*, bar graph (upper panel) showing the relative expression of SurM:SDx3M (mean $\pm$ S.D.; $n = 3$). GAPDH was used as a constitutive gene. RT-PCR analysis (lower panel) of M isoforms in HeLa cells transfected with CTRL and different siRNAs targeting SRp40, SRp55, hnRNP F, hnRNP H1, and hnRNP H2. *E*, Western blot analyses of pulldown assays using WTL and DobML biotin-labeled RNA probes under non-saturating conditions; the probes were incubated with total HeLa protein; the input was total protein from HeLa cells (INPUT), and C-lane was the last wash. The pulled down proteins were detected using specific antibodies against Sam68 and ASF/SF2. The p values from one-way ANOVA (*D*) are reported. *, $p < 0.05$; ***, $p < 0.001$; n.s. not significant.

affected the splicing of the SDx3M isoform, thus decreasing the relative ratio of both isoforms (Fig. 4C, lower panel). Thus, ExR1 and ExR3 could be antagonistic regulators of the ExR2 region.

Effect of *trans* elements on the regulation of M isoforms and endogenous survivin DEx3

To identify potential *trans* factors that bind to the *cis*-acting elements in exon 3, we took advantage of the previously performed *in silico* analyses. As the greatest expressional change was induced through the ExR2 mutation, we first focused on this region, which presents three potential binding candidates, Sam68 (also called KHDRBS1), hTra2B, and hnRNP G (Fig. 4A). To ascertain if any of these proteins are *bona fide* regulators, we carried out pulldown assays using two 17-bp RNA probes containing a wild-type or a mutated version of this region (Fig. 5A). As shown in Fig. 5B, Sam68 bound to the wild-

type probe but not to the mutated version, indicating that this mutation affects the binding of Sam68 to this region; hTra2B and hnRNP G did not show affinity for any of the tested RNA probes. These results confirm that Sam68 specifically binds to the UAAAAAGCAU sequence within the ExR2 region of exon 3, suggesting its involvement in regulating AS of the survivin DEx3 gene.

Because we previously found that mutation of the surrounding ExR1 and ExR3 regions produced an inhibitory effect on the expression of the SDx3M isoform and a possible antagonistic effect on the ExR2 region, we designed a double mutant RNA probe covering both regions. We reasoned that a mutation in the ExR1 and ExR3 regions would prevent the binding of any *trans*-acting factor, allowing Sam68 to freely bind to the ExR2 region, as it would not compete with another factor. Our previous *in silico* analyses showed that the SRp40, SRp55, hnRNP F, hnRNP H1, and hnRNP H2 factors were most likely to bind in

these two regions. To determine whether any of these factors mediates the observed effects of ExR2, we designed different siRNAs targeting these *trans* factors (Fig. 5C), which were then co-transfected with the M into HeLa cells. The siRNAs had little or no effect on the ratio of the two isoforms; therefore, these proteins probably do not regulate survivin AS (Fig. 5D).

The *in silico* analyses of the ExR1 and ExR3 regions showed that the ASF/SF2 factor presents one of the highest probabilities of binding in these regions. A previous report showed that this factor is able to bind to survivin mRNA, inducing its protein overexpression by affecting its mRNA translation (25). Thus, we performed a pulldown assay to determine whether ASF/SF2 binds to these regions. For this purpose, we designed two RNA probes, WTL and DobML. The first included the first 32 bp of exon 3, whereas the DobML probe contained the double mutation described above (Fig. 5A), which should prevent the binding of ASF/SF2. Western blotting showed that ASF/SF2 bound to the WTL probe but not to the DobML probe (Fig. 5E), indicating that ASF/SF2 binds to the ExR1 and ExR3 regions. We also tested whether the binding of ASF/SF2 to the ExR1 and ExR3 regions affects the binding of Sam68 to the ExR2 region, thereby altering the ratio of SurM and SDx3M. Pulldown and Western blot analyses demonstrated that the binding of ASF/SF2 to these regions did not affect the binding of Sam68 (Fig. 5E), excluding its possible participation in exon 3 skipping and survivin AS. Therefore, additional factor(s) are expected to be involved in the antagonistic actions of ExR1 and ExR3.

Finally, to further support the participation of Sam68 in the AS of survivin DEx3 mRNA, an siRNA (siSAM1) that targets Sam68 was transfected into HeLa cells (Fig. 6A). TaqMan and Western blot assays revealed that Sam68 depletion significantly altered the AS of the endogenous survivin DEx3 mRNA, decreasing the expression of this isoform (Fig. 6B). This result is consistent with the effect of the Mut2Ex3 construct, which affected the Sam68 splicing site and decreased the expression of the SDx3M isoform.

To further support our results in a more physiological model, exon 3 in endogenous survivin was mutated using the CRISPR/Cas9 technique (26, 27) (Fig. 6C). Two clones (CRISPRRH1 and CRISPRRH2) were created by mutating exon 3 in the same position as in the Mut2Ex3 construction mentioned above (Fig. 6D). This mutation affects the Sam68 binding site, which in previous experiments increased the inclusion of exon 3 and therefore decreased the expression of the SDx3M isoform (Fig. 4B). As expected, CRISPRRH1 and CRISPRRH2 cells presented a higher exclusion of exon 3, with a concomitant decrease in the expression of endogenous survivin DEx3 (Fig. 6E, *upper* and *lower* panels). We next evaluated whether siSAM1 had an effect on survivin DEx3 expression in a mutated ExR2 background using the CRISPRRH1 and CRISPRRH2 cells. [Supplemental Fig. S1A](#) shows no significant differences between the CRISPRRH1/2 cells and the CRISPRRH1/2 cells transfected with the siSAM1 siRNA. These results suggest that the Sam68 binding site is indeed responsible for survivin AS after Sam68 experimental modulation.

We also evaluated whether Sam68 overexpression affects the expression of survivin DEx3. Fig. 6F shows a reproducible

increase in survivin DEx3 mRNA following Sam68 overexpression (*lower panel*), as opposed to a very small change in expression of the main survivin isoform (not shown). As expected, Sam68 overexpression modulated AS of the *bclx* gene, as reported previously, but not Smac alternative splicing, a negative control ([supplemental Fig. S2, A and B](#)).

Finally, to explore the possible physiological impact of the putative Sam68 binding site, we performed xenotransplant dilution assays, in which we observed increased tumorigenicity in zebrafish embryos injected with CRISPRRH cells *versus* control (*Ctrl*) cells. (Fig. 6G). Similar results were found using colony formation assays ([supplemental Fig. S3A](#)) and soft agar colony assays ([supplemental Fig. S3B](#)) in cells transfected with siSAM1, further supporting our data.

In conclusion, our results suggest that Sam68 specifically binds to a 22-bp region within exon 3 to promote its exclusion, thereby increasing the expression of survivin DEx3 mRNA.

Discussion

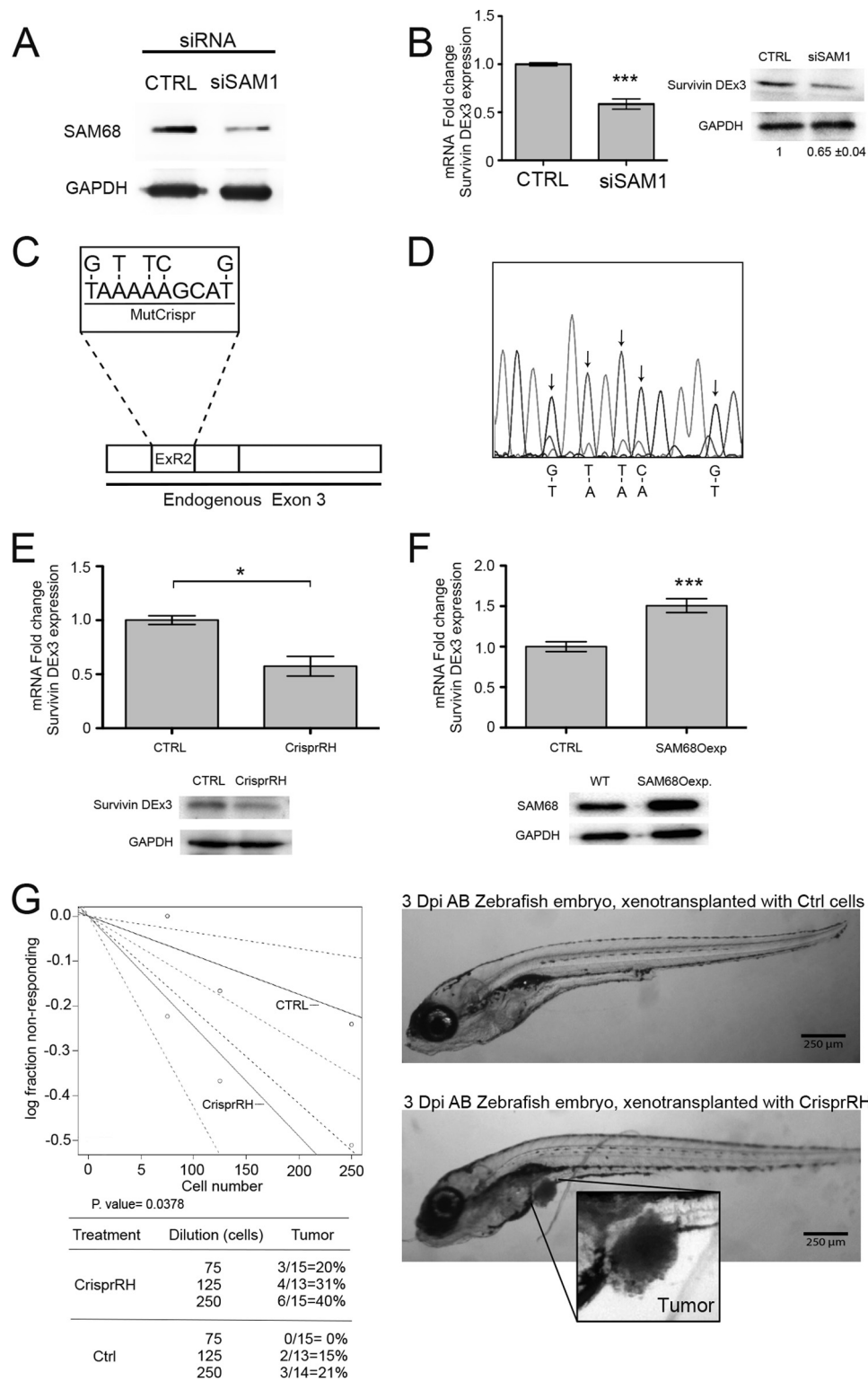
The appearance and deregulation of new transcripts because of AS play a crucial role in tumor development and patient survival because of their involvement in various carcinogenic processes, such as proliferation, apoptosis, and metastasis (28). Thus, understanding the molecular mechanisms that regulate AS will open new avenues for the development of novel splicing modulation strategies (29). Notably, *de novo* expression of anti-apoptotic survivin is a nearly universal feature in cancer, including breast, colon, lung, pancreatic, gastric, and bladder cancers, supporting a role in cancer development. Overexpression of this protein has been implicated in various processes associated with tumor progression, metastasis, and angiogenesis. Survivin is a key anti-apoptotic protein and is involved in mitosis, especially as part of the chromosomal passenger complex, which regulates chromosome segregation and cytokinesis (10, 30). In addition to survivin, five other isoforms generated through alternative splicing have been identified as follows: survivin DEx3, survivin 2B, survivin 2 α , survivin 3B, and the recently described survivin 2B+32 (13). These isoforms present differential expression that depends on the type and stage of cancer. As a consequence, some isoforms are better diagnostic or prognostic markers than survivin, pointing toward specific regulation and roles in each type of tumor. A clear example is survivin DEx3. This isoform is generated by the loss of exon 3 and possesses a new carboxyl terminus, and it exhibits nuclear and mitochondrial localization that is not observed for other isoforms. Its anti-apoptotic features and specific overexpression in thyroid cancer, breast cancer, ovarian cancer, and glioblastoma make survivin DEx3 a better diagnostic marker than survivin in these tumors.

In the present work, we identified several important regions that regulate survivin AS. For this purpose, we constructed an M that contains the minimum elements required to undergo exon 3 splicing. We then generated a series of deletions to identify possible *cis*-elements involved in AS. Deleting 200 bp upstream or 100 bp downstream of exon 3 increased exon exclusion, as reported for other genes (23). Using a similar approach, we then analyzed possible regulatory elements present in exon 3. Deleting the first 22 bp of exon 3 (Ex3 Δ 1 region)

Survivin DEx3 alternative splicing regulation

had a dramatic effect on the ratio of the two isoforms, favoring the exclusion of exon 3 and, consequently, increased expression of the SDx3M isoform at the expense of the SurM isoform. The second and third deletions also increased the expression of the SDx3M isoform, but in this case, SurM expression remained similar to the control, possibly because of the presence of a site or sites with an auxiliary role in exon 3 recognition. Because the

most remarkable shift in the ratio of the two isoforms was caused by the Ex3Δ1 deletion, we analyzed the loss of function of recognition elements by introducing mutations in the potential binding sites of *trans* factors. For this purpose, we divided these 22 bp into the three subregions or binding clusters, designated ExR1, ExR2, and ExR3, based on the potential factors that bind these sequences. Because of the size of the subregions



and factor clustering, mutations in each subregion were expected to prevent the binding of several putative *trans* factors. Unexpectedly, we found that a mutation in the second cluster (ExR2) had a significant effect on the ratio of the two isoforms, increasing the expression of SurM. As we did not detect an effect when either of the surrounding regions (ExR1 and ExR3) were mutated, we hypothesized that these regions in combination have an antagonistic effect on the middle region, perhaps via steric hindrance of an unknown factor. Hence, we generated a double mutant construct (DobMutEx). As expected, DobMutEx affected the ratio of the two isoforms, thus increasing the exclusion of the exon and increasing SDx3M expression, similar to what we observed for the Ex3Δ1 deletion. Therefore, we identified a possible new exonic splicing silencer *cis*-acting element in the first 22 bp of survivin exon 3. The next step was to identify the possible *trans*-acting factor(s) that may interact with this region to regulate the AS of survivin DEx3 mRNA.

Binding prediction programs identified SRp40, SRp55, hnRNP H1, hnRNP H2, hnRNP F, Sam68, hnRNP G, hTra2β, and ASF/SF2 as potential regulators. Interestingly, these factors are involved in regulating the AS of a variety of genes involved in cancer (4, 31–33). We performed pulldown and siRNA assays to identify which of these factors were involved in regulating survivin AS. We also used RNA probes that contained mutations in the binding sites for specific RNA binding proteins (RBPs), such as Sam68, hnRNP G, hTra2β, and ASF/SF2, for pulldown analyses. Through these analyses, we identified Sam68 as a *bona fide* regulator of survivin DEx3 mRNA AS. Several reports have shown that Sam68 exhibits important oncogenic functions through splicing modulation of cancer genes. Sam68, the best-characterized member of the STAR proteins, is implicated in cell cycle progression, apoptosis, and tumorigenesis (34). Sam68 plays an important role in regulating the AS of specific mRNAs, acting as a splicing repressor or enhancer. Sam68 regulates the AS of CD44 (35) and promotes the inclusion of exon 5 through phosphorylation and ERK1/2 signaling in mouse T lymphoma cells (36). In addition, Sam68 is required for the AS of human papillomavirus (HPV), promoting exon E6 inclusion (37). Moreover, the Sam68 protein is involved in the epithelial to mesenchymal transition during tumor metastasis by means of regulating the AS of ASF/SF2 (38). Furthermore, Sam68 overexpression plays a role in apoptosis by modulating the AS of BCL-X isoforms in cooperation with hnRNP A1 in prostate and breast carcinoma (39). This finding was consistent with our previous results based on the

Mut2Ex3 mutation, which modified the Sam68 binding site and consequently altered the proportion of the isoforms to favor the SurM isoform (decreasing exon 3 exclusion). Other factors such as hnRNP G and hTra2β did not bind to any of the probes, excluding their involvement in the regulation of survivin DEx3 mRNA AS.

To further support the role of Sam68 in survivin AS, an siRNA (siSAM1) was designed to inhibit Sam68 expression and analyze its effect on endogenous survivin DEx3 mRNA. As expected, we observed a reproducible decrease in the expression of endogenous survivin DEx3.

To explore if the Sam68 binding site is important for regulating the AS of endogenous survivin exon 3, we designed two mutant cell lines using a CRISPR/Cas9 approach. Our results showed that mutating the Sam68 binding site resulted in a decreased survivin DEx3. We then evaluated if the alteration of the binding site or the inhibition of Sam68 were physiologically relevant. For this, we performed xenotransplant dilution assays and colony formation assays. As expected, we observed an increase in tumorigenicity and in colony-forming ability. Therefore, these results suggest that decreased survivin DEx3 expression increases tumor formation. This result correlates with our previous report (15) in which the overexpression of survivin DEx3 decreased the ability of HeLa cells to form colonies, as well as the capacity to form tumor spheroids. Although not explored, an increase in the number of tumor-initiating cells may underlie these effects, as has been reported previously (40, 41).

One possible model to explain our data is that the binding of Sam68 at survivin exon 3 determines whether the splicing machinery recognizes exon 3. If Sam68 is absent at this site, perhaps because of the binding of a putative unknown factor to the adjacent regions, then the 5' splice site of exon 3 will be recognized and included. Conversely, if Sam68 is present, the 5' site of exon 3 will be blocked, and the exon will not be recognized and will not be included in the transcript (Fig. 7). A previous study showed that ASF/SF2 can bind to survivin mRNA without interfering with its splicing but does affect mRNA translation (25). Interestingly, we also found that ASF/SF2 bound to exon 3 of survivin in two regions surrounding the Sam68 binding site. We hypothesized that ASF/SF2 binding may exclude Sam68 from its consensus site, but we were unable to identify interference among these factors. These results suggest that another factor or factors compete with Sam68 for the recognition of exon 3, resulting in a balance of the expression of the two isoforms.

Figure 6. Sam68 regulates the alternative splicing of endogenous survivin DEx3 mRNA. *A*, Western blot analyses of HeLa cells transfected with an siRNA against Sam68, showing the resulting knockdown efficiency. GAPDH expression was used as an internal control. *B*, bar graph (*left panel*) showing the relative fold-change in endogenous survivin DEx3 mRNA expression determined via qPCR after siRNA targeting Sam68 was transfected into HeLa cells (mean $\pm$ S.D.; $n = 4$). GAPDH was used as a constitutive gene. Western blot analyses (*right panel*) of survivin DEx3 in HeLa cells transfected with a siRNA against Sam68. A representative image is shown, and the densitometry values (mean $\pm$ S.D.) from three replicates appear under the image. *C*, schematic diagram of the endogenous exon 3, as well as the position of the mutation in the ExR2 region. *D*, electropherogram showing the mutated bases in endogenous survivin exon 3 after CRISPR/Cas9 mutation. The arrows indicate the position of the mutated bases. The combined peaks in each position show a heterozygous locus and lower allele fraction in the cultivated cells. *E*, bar graph (*upper panel*) showing the relative expression of endogenous survivin DEx3 mRNA expression determined by qPCR in HeLa Cas9 cells and CRISPRRH cells ($n = 3$). GAPDH was used as a constitutive gene. Western blot analyses (*lower panel*) of survivin DEx3 in CRISPRRH cells. *F*, bar graph (*upper panel*) showing the relative expression of endogenous survivin DEx3 mRNA determined by qPCR after Sam68 overexpression (Sam68Oexp) in HeLa cells. Mean $\pm$ S.D.; $n = 3$. Western blot analyses (*lower panel*) of Sam68 overexpression. *G*, xenotransplant dilution assays using a zebrafish model. *Right panel*, tumors developed in zebrafish embryos at 3 dpi with HeLa CRISPRRH cells or HeLa Cas9 control (Ctrl) cells. *Left panel*, limiting dilution analysis obtained with the extreme limiting dilution assays (ELDA) software. Plot shows the percentage of embryos injected with CRISPRRH1 or Ctrl cells with abdominal tumors at 4 dpi. The *p* values from Student's *t* test (*B*, *E*, and *F*) are reported. *, $p < 0.05$; ***, $p < 0.001$; n.s., not significant.

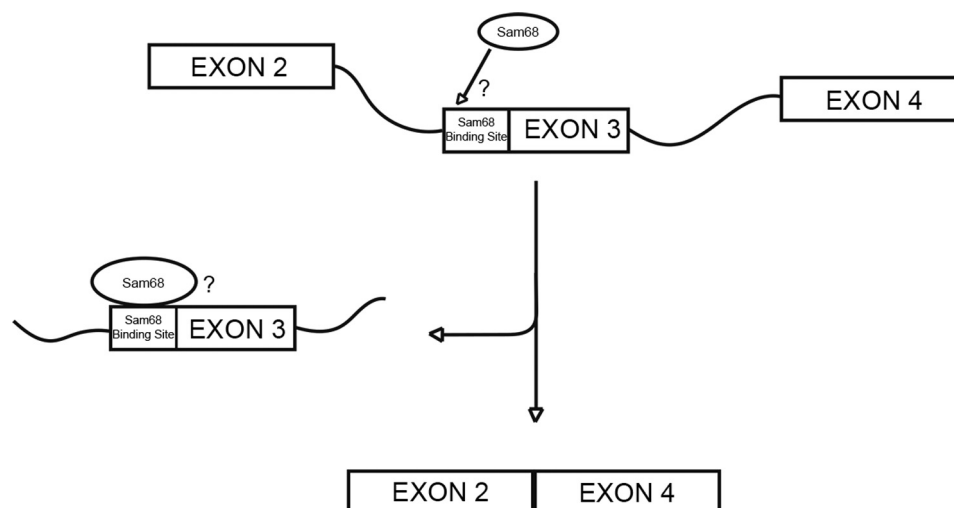


Figure 7. A possible model of the regulation of exon 3 skipping by Sam68 is shown. When Sam68 is expressed and bound to exon 3 in the first 22 bp, it blocks the recognition of exon 3, causing skipping of exon 3. When Sam68 is down-regulated, posttranslationally modified, or excluded through steric hindrance by an unknown factor, exon 3 is recognized and included in the mRNA.

In conclusion, this is the first work that provides evidence that inhibiting Sam68 or disrupting its *cis*-acting elements modulates survivin DEx3 splicing. Delving into the mechanism of survivin DEx3 AS is crucial because of the importance of this isoform in cancer progression and as a possible diagnostic and prognostic marker.

Experimental procedures

Minigene construction

We constructed an M via PCR. As a template, we used human genomic DNA obtained from peripheral blood that was purified with the QIAamp DNA Blood Kit (Qiagen, Valencia, CA). The M was amplified in three separate reactions. The first reaction amplified 550 bp of the *survivin* gene, including exon 2 and part of intron 2. The following primers were used: (forward) 5'-CGGGCTGCCAAGCTTACTCAGAGCTG-3' and (reverse) 5'-AGCCTCCGGATCCGTGAAGTGCACACTT-3', which contained HindIII and BamHI sites. The second round of PCR amplified 1,551 bp of the *survivin* gene, including exon 3 and parts of introns 2 and 3, using the primers (forward) 5'-TTCTCTGCCCTGGATCCTTACAGTGGGC-3' and (reverse) 5'-AATAAAAAGAATTCCTGAGGCCAGGTGTG-3', which contained BamHI and EcoRI sites. Finally, the third round of PCR amplified a 470-bp region of the *survivin* gene, including part of intron 3 and part of exon 4, with the primers (forward) 5'-AGTAGGAATTCGGGAGCACCCAGCGC-3' and (reverse) 5'-GAATACTCGAGGGAAGTGGTGCAGCCACTC-3'; these primers contained EcoRI and XhoI sites. The M was cloned into the plasmid pcDNA 3.1(+) (Invitrogen) in three steps. All fragments were verified by sequencing. All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA).

Deletions

M deletions were performed using the QuikChange Lightning Kit (Agilent Technologies, Santa Clara, CA) as indicated in a previously published procedure (42), with minor modifications. The PCR program was modified to 18 cycles (95 °C for 20 s, 54 °C for 10 s, and 68 °C for 4 min 30 s) for exon deletions

and 18 cycles (95 °C for 20 s, 55 °C for 10 s, and 68 °C for 4 min 30 s) for intron deletions. Fourteen deletions were introduced into the introns via PCR, including seven deletions upstream of exon 3 (Δ I1, Δ I2, Δ I3, Δ I4, Δ I5, Δ I6, and Δ I7) and seven deletions downstream of exon 3 (Δ I8, Δ I9, Δ I10, Δ I11, Δ I12, Δ I13, and Δ I14). Each deletion was 100 bp long, covering 700 bp on both sides of exon 3. The regions 60 bp upstream of exon 3 and 10 bp downstream of exon 3 were conserved to retain the splicing consensus sites. In exon 3, three deletions were generated with the same methodology. We divided exon 3 into three sections as follows: the first 22 bp from the 5' side were deleted in the Ex3 Δ 1 construct, whereas the next 49 bp were deleted in Ex3 Δ 2 and finally 37 bp in Ex3 Δ 3. These sections were selected following *in silico* analysis of splicing elements (see "Identification of intronic and exonic *cis* elements that regulate survivin AS"). The splicing consensus sites of the exon 3'-GA and 5'-TG were conserved, in addition to 5 bp on each side of exon 3. All of the primers used in this procedure are described in [supplemental Table S1](#). All deletions were verified by Sanger sequence analysis.

Mutations

Three site-directed mutations were generated in exon 3, which were designated Mut1Ex3, Mut2Ex3, and Mut3Ex3. The mutations were generated via PCR using the QuikChange Lightning Kit (Agilent Technologies) according to the manufacturer's protocol. The PCR program was modified to 18 cycles (95 °C for 20 s, 55 °C for 10 s, and 68 °C for 4 min 30 s). All mutations were verified by Sanger sequencing. For the generation of MutDobEx, which is a double mutation of the putative *cis*-acting elements, we used the template of the Mut1Ex3 construct and inserted the mutation into the Mut3Ex3 construct through PCR, using the aforementioned PCR program. All of the primers used in this procedure are listed in [supplemental Table S1](#).

Cell culture and transfection

Cervical adenocarcinoma (HeLa) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS (ATCC, Manassas, VA). HeLa cells (6.8×10^4) were

cultured in 24-well plates and transfected with 800 ng of plasmid DNA from each of the constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Then, 48 h after transfection, RNA was extracted using the RNeasy Mini Kit (Qiagen) and TRIzol (Invitrogen). Three biological replicates were performed for each condition.

Soft agar colony assay

HeLa cells transiently transfected with Sam68 siRNA (siSAM1) or control siRNA (CTRL) were suspended in 0.33% agar with DMEM containing 5% fetal bovine serum. Then, 5.5×10^3 cells were seeded in triplicate in a 6-well plate onto a 0.5% agar base. Cultures were maintained at 37 °C with 5% CO₂ for 13 days. Colonies were stained with 0.005% crystal violet and photographed on a ChemiDoc MP Imaging System (Bio-Rad) using Image Lab software. Images were quantified using the ColonyArea plugin of ImageJ. Two independent experiments were performed.

Colony formation assay

HeLa cells transiently transfected with Sam68 and control siRNAs were seeded at 100 cells per well in a 6-well plate in triplicate. Cultures were followed for 12 days. Colonies were fixed with 70% ethanol and stained with 0.1% crystal violet as described (43, 44). Stained plates were photographed on a ChemiDoc MP Imaging System (Bio-Rad) using Image Lab software. For colony quantification, the ColonyArea plugin of ImageJ was used. Two independent assays were performed.

RT-PCR

PCR was performed to measure the relative abundance and expression of the M transcripts. T7 and BGH primers were used, as both recognize regions in the pcDNA 3.1 vector, avoiding endogenous survivin mRNA and its isoforms. One microgram of RNA was used for the reverse transcription (RT) reaction with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For PCR, each reaction contained 200 μM dNTP, 0.2 μM primers, and 1.25 units of Taq Gold DNA Polymerase (Roche), and the PCR program consisted of 35 cycles of 95 °C for 30 s, 47 °C for 30 s, and 72 °C for 30 s. The PCR products were analyzed via 2% agarose gel electrophoresis.

For RT-PCR analyses of endogenous survivin, the following primers were used: (forward) 5'-GCCATGAATTCATGGGT-GCCCCGACGTTGC-3' and (reverse) 5'-AGCTCTCT-AGAGAGGCCTCAATCCATGGCA-3' (Integrated DNA Technologies). The PCR conditions consisted of a pre-step of 3 cycles of 95 °C for 30 s, 68 °C for 30 s, and 72 °C for 30 s, followed by 35 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. Quantitative PCR (qPCR) assays for endogenous survivin and survivin DEx3 mRNA were performed using TaqMan Universal PCR Master Mix and TaqMan probes for survivin mRNA (Hs00977612_mH), survivin DEx3 mRNA (Hs03043576_m1), and GAPDH mRNA (Hs00266705_g1) (Applied Biosystems) under the recommended PCR conditions (95 °C for 10 m, 95 °C for 15 s, 60 °C for 30 s, for 40 cycles). Amplification of the SDx3M isoform was performed using SYBR Select Master Mix, and the following primers were used: (forward) 5'-GAC-

CCCATGCAAAGGAAAC-3' and BGH primer, and the PCR program consisted of 40 cycles (95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s). All assays were carried out on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems); three biological replicates were performed for each condition. The Sam68 overexpression plasmid was obtained from OriGene Technologies Inc. (Rockville, MD) and transfected using Lipofectamine 2000. For the RT-PCR analyses of endogenous BCL-X, the following primers were used: (forward) 5'-ATG-TCTCAGAGCAACCGGGAGCTG-3' and (reverse) 5'-TCATTTCCGACTGAAGAGTGAGCC-3' (Integrated DNA Technologies). The PCR conditions consisted of 40 cycles of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. For endogenous SMAC the following primers were used: (forward) 5'-GCGCG-GATCCATGGCGGCTCTGAAGAGTTGGCT-3' and (reverse) 5'-AGCTCTCTAGACTCAGGCCCTCAATCCTCACGC-3' (Integrated DNA Technologies). The PCR conditions consisted of a pre-step of 3 cycles of 95 °C for 30 s, 72 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 68 °C for 30 s, and 72 °C for 1 min.

siRNA transfection

The sequences used for siRNA are described in [supplemental Table S1](#). We designed five siRNAs (Integrated DNA Technologies) to inhibit the expression of the SRp55, SRp40, hnRNP H1, hnRNP H2, hnRNP F, and Sam68 genes, as well as an siRNA targeting the Luciferase gene as a control (CTRL). Each siRNA was cloned into the pSIREN-retroQ vector (Clontech), and cells were transfected using the FuGENE HD Transfection Reagent (Promega, Madison, WI) or Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. After 72 h, RNA and protein were extracted for downstream analysis.

Computational analysis of cis and trans elements

Three different programs were used to predict putative *cis* and *trans* AS elements: splicing Rainbow (discontinued in 2012), ESEfinder 3.0 (45), and SpliceAid 2 (46). WebLogo was employed to align the first 22 bp of exon 3 from 15 different species (47).

Whole-cell lysate and Western blot analyses

Cells were lysed with the M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) and supplemented with a protease inhibitor mixture (Sigma), and total proteins were resolved via 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA). The primary antibodies used in these assays were anti-Sam68 (C-20) (Santa Cruz Biotechnology, Dallas, TX), anti-SF2/ASF (96) (Santa Cruz Biotechnology), anti-RBMX (hnRNP G) (H-80) (Santa Cruz Biotechnology), anti-TRA2B (Abcam, Cambridge, UK), anti-survivin DEx3 (Abcam), and anti-GAPDH (FL-335) (Santa Cruz Biotechnology).

RNA pulldown assays

RNA probes were purchased from Integrated DNA Technologies, and HeLa protein was used for the assays. A Pierce Magnetic RNA-Protein Pull-down Kit (Thermo Scientific) was used according to an established protocol, with slight modifications.

Survivin DEx3 alternative splicing regulation

For the ligation of desthiobiotinylated cytidine bisphosphate to the 3' end of the RNA probe, 60 pmol of the RNA probe was used. Protein and RNA were incubated for 2 h under orbital rotation. The RNA-binding protein complex was washed four times with 100 mM KCl (48) and then four times with 1× wash buffer. The sequences of the RNA probes were as follows: (WTE_{x3}) ACAUAAAAAGCAUUCGU, (M2Ex₃) ACA-GAUAUCAAGUCGU, (WTL) AGAGAGGAACAUA- AAAGCAUUCGUCCGGUUG, and (DoBML) AGCCA-GAUAUCAAAAAAGCCUGAUUAGAUUG. All RNA probes were obtained from Integrated DNA Technologies.

Mutation of the endogenous survivin exon 3 by CRISPR/Cas9

To mutate exon 3 of endogenous survivin, the Alt-R CRISPR/Cas9 System (Integrated DNA Technologies) was used, with some modifications. A guide RNA (crRNA) was designed using the CRISPR Design software (<http://crispr.mit.edu>).⁵ The sequence used in the experiments was GAACAUAA-AAAGCAUTC; the tracrRNA and crRNA oligonucleotides were obtained from Integrated DNA Technologies. The lenti-Cas9-Blast was a gift from Feng Zhang (Addgene plasmid no. 52962). The RNA oligonucleotides were mixed in an equimolar concentration to create a final complex concentration of 3 μM. For the homologous recombination (HR) process required to create the mutant, an ssDNA oligo targeting exon 3 of the survivin gene at 100 nM concentration was used. This oligonucleotide contained a mutation to affect the binding of Sam68. The sequence used was TTGTTTTGATTTTCTAGAGAGG-AACAGATATCGCAGTCGTCGGTTGC.

HeLa cells were stably transfected with the Cas9 plasmid using the Lipofectamine 2000 transfection reagent according to manufacturer's protocol (Invitrogen) and selected using 6 μg/ml of blasticidin. RNAiMax (Invitrogen) was used to transfect the complexed tracrRNA:crRNA and the ssDNA oligonucleotide according to the manufacturer's protocol. The cells used to form single-cell colonies were named CRISPRRH1 and CRISPRRH2. These clones were validated by Sanger sequencing to verify the presence of the mutations.

Xenotransplant assays

The zebrafish AB wild-type strain was kindly donated by Dr Hilda Lomely from IBT-UNAM. Adult zebrafish were maintained at 28 °C in standard conditions (pH 7.2–7.4; 14 h on and 10 h off light cycle). Fish embryos were obtained by natural crosses and then maintained at 28 °C in embryo medium (49, 50). Cell xenotransplants were carried out in the embryonic yolk sacs of embryos 2 days post fertilization (dpf) that were previously dechorionated and anesthetized with tricaine (MS-222; Sigma). Both CRISPRRH1 cells and control cells were resuspended at a concentration of 4×10^6 cells/100 μl, and 75, 125, or 250 cells were microinjected for each group. To study tumorigenic capacity, ~15 embryos were microinjected with each group of cells and monitored for 4 days post injection (dpi) for tumor formation. *In vivo* limiting dilution assays were analyzed using the extreme limiting dilution assays (ELDA) soft-

ware (51) which employs a generalized linear model used to compare active cell frequencies in cell populations.

Statistical analysis

Student's *t* test was used to compare two conditions. A one-way analysis of variance (ANOVA) with Dunnett's post hoc test was used to analyze three or more conditions. Standard deviation bars are shown in all graphs, and *p* < 0.05 was considered statistically significant. All analyses were performed using GraphPad Prism version 5.00 (GraphPad Software, La Jolla, CA).

Author contributions—J. G.-C. conducted most of the experiments, analyzed the results, and wrote the article. C. G.-T. conducted replicates and independent experiments. V. M. coordinated and analyzed the replicated data and independent experiments. G. C.-C. analyzed the data and wrote the paper. C. Z. performed zebrafish *in vivo* experiments. J. M.-Z. coordinated the study, analyzed the data, and wrote the paper with J. G.-C. and G. C.-C.

Acknowledgments—We thank Dr. Hilda Lomely from IBT-UNAM for donating the zebrafish AB wild-type strain.

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