KDM3A regulates alternative splicing of cell-cycle genes following DNA damage

Mai Baker[1], Mayra Petasny[1], Mercedes Bentata[1], Gillian Kay[1], Eden Engal[1], Yuval Nevo[1], Ahmad Siam[1], Sara Dahan[1] and Maayan Salton[1,*]

[1]Department of Biochemistry and Molecular Biology, the Institute for Medical Research Israel Canada, Faculty of Medicine, Hebrew University of Jerusalem, Jerusalem 91120, Israel

*To whom correspondence should be addressed. Tel: +972 26758814; Email: maayan.salton@mail.huji.ac.il

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ABSTRACT

Changes in the cellular environment result in chromatin structure alteration, which in turn regulates gene expression. To learn about the effect of the cellular environment on the transcriptome, we studied the H3K9 de-methylase KDM3A. Using RNA-seq, we found that KDM3A regulates the transcription and alternative splicing of genes associated with cell cycle and DNA damage. We showed that KDM3A undergoes phosphorylation by PKA at serine 265 following DNA damage, and that the phosphorylation is important for a proper cell cycle regulation. We demonstrated that SAT1 alternative splicing, regulated by KDM3A, plays a role in cell cycle regulation. Furthermore we found that KDM3A's demethylase activity is not needed for SAT1 alternative splicing regulation. In addition, we identified KDM3A's protein partner ARID1A, the SWI/SNF subunit, and SRSF3 as regulators of SAT1 alternative splicing and showed that KDM3A is essential for SRSF3 binding to SAT1 pre-mRNA. These results suggest that KDM3A serves as a sensor of the environment and an adaptor for splicing factor binding. Our work reveals chromatin sensing of the environment in the regulation of alternative splicing.

INTRODUCTION

Splicing of precursor mRNA (pre-mRNA) is an important regulatory step in gene expression. The process of alternative splicing enables the production of protein isoforms from a single gene, isoforms that can have different functions, all contributing to the cell's diverse tasks[1]. Traditionally the regulation of alternative splicing was attributed to regulatory elements in the pre-mRNA. Recently we and others have shown the link between chromatin structure and epigenetic modifications in splicing regulation[2-10]. The data linking chromatin and alternative splicing is not enough to explain the physiological causes and consequences of chromatin-mediated changes in alternative splicing. Furthermore, a major unanswered question in the field is how, and to what extent, environmental changes shape the cell transcriptome via modulation of alternative splicing.

Cellular environment has been shown to modulate splicing in several ways. The main one is the induction of post-translational modifications of splicing factors. Phosphorylation of SR splicing factors affects their protein-protein interactions as well as regulating protein activity. A specific example is activation of the Fas receptor leading to dephosphorylation of SR proteins that promote pro-apoptotic isoforms of two key regulators, Bcl-X and caspase-9[11]. Another example is cell-cycle-dependent splicing patterns, which are also modulated by SR protein phosphorylation by specific kinases and phosphatases such as NIPP1[12]. Another mode of action for alternative splicing regulation following cellular environmental changes could be by modulating chromatin, as changes in the environment are sensed by cells and transduced into changes in gene expression, leading to specific cellular responses.

The histone demethylase KDM3A has been shown to respond to the environment to alter transcription patterns. KDM3A's specific demethylase activity is on H3K9me1 and H3K9me2 catalyzed by histone methyltransferases EHMT2/G9a and EHMT1/GLP[13,14]. Unlike H3K9me3, which is mostly a repressive mark present in heterochromatin regions, H3K9me2 is mainly on euchromatin[13,14]. The regulation of H3K9me2 is dynamic and KDM3A is known to be regulated post-translationally in multiple ways and thus it is suitable to be a responsive sensor for the environment.

KDM3A was shown to be phosphorylated in response to cold temperatures in adipose tissue and promote a specific expression pattern by demethylating promoters of genes important for chronic adaptation to cold stress[15]. In addition to cold shock, KDM3A is also phosphorylated on serine 265 following β-adrenergic stimulation. Interestingly, the modification promotes a scaffold function of KDM3A separate to its demethylase activity[16]. Serving as a scaffold for acetyltransferases present in enhancers and promoters allows transcription of key metabolic

62 genes[16]. Under heat shock conditions phosphorylation of KDM3A on serine 264 promotes
63 transcription of specific target genes[17]. Another response by KDM3A is to pressure-overload
64 by cardiomyocytes which promotes transcription of genes with pro-fibrotic activity[18]. In
65 addition, KDM3A responds to IL-6, androgen receptor and hypoxia[19-21].

66 KDM3A's pivotal role in transcription suggests that its misregulation could be a cause of
67 disease. Indeed upregulation of KDM3A is related to tumorigenesis of colorectal cancer cell
68 migration and invasion[22]. This function of KDM3A is attributed to its transcription of c-*Myc*,
69 cyclin D1, and *PCNA*[23,24]. In addition regulation of androgen receptor and estrogen receptor
70 transcription by KDM3A position it as a central player in prostate and breast cancer [20,25-29].

71 The role of H3K9me2 modification in alternative splicing was demonstrated by us and others.
72 While H3K9me2 modification in promoter regions have been shown to cause gene silencing,
73 the same modification in the intragenic region of genes was shown to alter alternative
74 splicing[25,30-38]. The connection of the H3K9 methylation and the spliceosome was shown to be
75 via an adaptor system containing a chromatin reader, which is one of the three HP1 proteins
76 known to bind H3K9 methylation, HP1α, β and γ[39]. The chromatin reader in turn will bind a
77 splicing factor that will recruit or block the spliceosome to promote a specific event of
78 alternative splicing[30].

79 Here we explored KDM3A's role in the cellular response to the changing environment. To this
80 end we conducted RNA-seq following silencing of KDM3A and discovered a functional
81 group of cell-cycle genes to be regulated in both expression and alternative splicing. We
82 identified protein kinase A (PKA) as the kinase that phosphorylates KDM3A serine 265
83 following DNA damage. This phosphorylation is important for KDM3A's function as a
84 scaffold joining the SWI/SNF complex and the splicing factor SRSF3 to modulate its targets.
85 We focused on one of its targets, SAT1, and showed that regulating its alternative splicing is
86 important for proper cell-cycle regulation following DNA damage. Our work exemplifies the
87 importance of alternative splicing in the cellular response to the cell's changing environment.

88

89 **RESULTS**

90 **KDM3A regulates transcription and alternative splicing of cell-cycle genes**

91 In order to learn how changes in the cellular environment result in chromatin structure
92 alteration, which in turn regulates gene expression, we silenced the prominent cellular sensor
93 histone demethylase KDM3A. Since KDM3A's role in breast cancer progression is well
94 established, we chose to use the breast cancer cell-line MCF7[27-29]. We began by characterizing

95 all KDM3A-dependent expression and alternative splicing events by means of deep RNA-
96 sequencing at a genome-wide scale using siRNA (Supplementary Fig. S1A). As expected of a
97 demethylase, we detected 1075 genes that are differentially expressed when KDM3A is
98 silenced (cut-off set at 1.5-fold); of these 3% were also alternatively spliced. Interestingly, we
99 found a further group of 777 genes that were alternatively spliced (Fig. 1A). This result
100 suggests that KDM3A is a regulator of alternative splicing and that alternative splicing
101 regulation by KDM3A is almost exclusively independent of its role as a transcription
102 regulator.

103 Among the 809 genes that were differentially spliced, we observed all types of alternative
104 splicing events: skipped exons (SE; 573 events in 477genes), alternative 5′ splice site (A5SS;
105 118 events in 109 genes), alternative 3′ splice site (A3SS; 100 events in 97 genes), mutually
106 exclusive exons (MXE; 119 events in 96 genes) and retained introns (RI; 97 events in 94 genes).
107 We chose to validate five genes that were indicated by RNA-seq to be regulated in alternative
108 splicing by KDM3A; of those four showed significant changes (Supplementary Fig. S1B-C).
109 Using the DAVID function annotation tool (DAVID, https://david.ncifcrf.gov/)[40,41], we
110 found that the list of differentially expressed genes was enriched for genes with functions in
111 cell cycle, cell migration and cell motility (Fig. 1B). The differentially alternative spliced genes
112 were enriched for genes involved in metabolic processes, DNA repair, cell cycle, and cellular
113 response to DNA damage (Fig. 1C). This result demonstrates that while KDM3A regulates
114 different genes in expression and alternative splicing, the functions of the genes it regulates
115 are similar. In addition these results suggest a new role for KDM3A following DNA damage.

116 The DNA damage response is a cellular system that maintains genomic stability. This system
117 activates an enormous amount of proteins that efficiently modulate many physiological
118 processes. The overall cellular response to DNA damage goes far beyond repair. It modulates
119 numerous physiological processes, and alters gene expression profiles and protein synthesis,
120 degradation and trafficking. One of its hallmarks is the activation of cell-cycle checkpoints
121 that temporarily halt the cell-cycle while damage is assessed and repaired [42].

122 **KDM3A is phosphorylated by PKA following induction of double-strand breaks**

123 KDM3A phosphorylation has been shown to occur following a change in temperature[16,17].
124 Heat stress induces KDM3A S264 phosphorylation[17], whereas cold stress promotes KDM3A
125 S265 phosphorylation[16]. In order to check whether KDM3A is phosphorylated following DNA
126 damage, we began by exploring mass-spec data using phospho-site[43]. We found that KDM3A
127 S265 is phosphorylated following treatment with etoposide, a topoisomerase II inhibitor that
128 primarily causes double-strand DNA breaks[44]. To validate this mass-spec result, we induced

129 double-strand DNA breaks in MCF7 cells with the radiomimetic drug neocarzinostatin (NCS).
130 We detected KDM3A S265 phosphorylation using an antibody specific for its phospho-S265[16].
131 Our result shows that KDM3A S265 is phosphorylated while the total amount of KDM3A does
132 not change following DNA damage (Fig. 2A).

133 We continued by asking which is the kinase that phosphorylates KDM3A. While the nuclear
134 kinase ataxia telangiectasia mutated (ATM) is the main kinase activated following double-
135 strand breaks[45], the motif in KDM3A S265 does not match ATM's preference (serine followed
136 by glutamine, SQ). The motif in fact suggests that the kinase is PKA[16]. While PKA is not a
137 bona-fide DNA damage kinase, it is known to phosphorylate ataxia telangiectasia and Rad3-
138 related protein (ATR) following single-strand breaks[46]. To check whether PKA
139 phosphorylates KDM3A on S265 following double-strand breaks, we incubated MCF7 cells
140 with NCS with or without PKA inhibitor. Our results show reduced phosphorylation of
141 KDM3A S265 following inhibition of PKA, indicating that PKA is KDM3A's kinase (Fig. 2B).

142 **More cells in S phase following silencing of KDM3A or mutation at its phosphorylation**
143 **site**

144 To examine KDM3A's role in cell cycle checkpoint following DNA damage, we silenced
145 KDM3A using siRNA and induced double-strand breaks with NCS. We found a mild increase
146 in percentage of cells in S-phase in cells silenced for KDM3A relative to control (Fig. S2A-C).
147 The slight change might result from redundancy of KDM3A function with other demethylases
148 such as KDM4C[47].

149 To check if phosphorylation of KDM3A S265 is important for S-phase checkpoint, we stably
150 expressed KDM3A with S265 mutated to alanine (S265A) or aspartic acid (S265D), mimicking
151 the non-phosphorylated or phosphorylated form, respectively (Supplementary Fig. S2D).
152 Comparing the cell cycle recovery in these cells to cells stably expressing WT KDM3A, we
153 found a higher percentage of cells in S-phase in S265A but not in S265D, suggesting that
154 KDM3A phosphorylation plays a role in cell cycle regulation (Fig. 2C and Supplementary Fig.
155 S2E). In addition, we monitored the cell cycle of MCF7 cells whose PKA was inhibited and
156 found a similar effect with a higher percentage of cells in S-phase (Fig. 2D and Supplementary
157 Fig. S2F). This supported the idea that PKA and KDM3A are in the same pathway in the
158 cellular response to DNA damage.

159 **SAT1, a KDM3A target in alternative splicing regulation**

160 To study KDM3A's role in cell cycle via alternative splicing, we chose to examine its target
161 gene SAT1 (Fig. S1C). SAT1 belongs to the acetyltransferase family and is a rate-limiting

162 enzyme in the catabolic pathway of polyamine metabolism. The acetylation of spermidine
163 and spermine by SAT1 regulates the cellular levels of free spermidine/spermine and is critical
164 since a decrease in their concentrations inhibits cell proliferation while an excess appears to
165 be toxic[48]. Thus the exact amount of SAT1 is important and regulated by transcription, mRNA
166 splicing, translation, and protein stability[14]. SAT1 has seven exons, and recently it was
167 reported that exon X (present between SAT1 exon 3 and 4) undergoes alternative splicing (Fig.
168 3A)[14]. Exon X codes for three stop codons and as a result the mRNA harboring exon X is a
169 target of the nonsense-mediated mRNA decay process[14]. This is an example of an alternative
170 splicing event that leads to a decrease in the total protein amount. Our RNA-seq followed by
171 validation demonstrated that silencing of KDM3A promoted SAT1 exon X inclusion
172 (Supplementary Fig. S1C and S3A), which decreased the amount of stable SAT1 mRNA[14].
173 Complementarily, overexpression of KDM3A in MCF7 cells promoted SAT1 exon X exclusion
174 (Supplementary Fig. S3B&C), and in the same way, following NCS we observed a slight
175 exclusion of SAT1 exon X that was abolished by inhibition of PKA (Supplementary Fig.
176 S3D&E). This result suggests that KDM3A phosphorylation is important for SAT1 alternative
177 splicing. To provide support for this, we monitored SAT1 exon X inclusion in our cells that
178 stably expressed either S265A or S265D. We found that overexpression of KDM3A in its
179 constitutively phosphorylated form promoted SAT1 exon X exclusion, similar to the
180 alternative splicing seen following DNA damage (Supplementary Fig. F&G). Next we
181 investigated the importance of SAT1 alternative splicing to cell cycle in MCF7. We silenced
182 SAT1, mimicking inclusion of exon X. We found a higher percentage of cells in S-phase
183 resembling the effect of silencing KDM3A (Fig. 3B and Supplementary Fig. S3H&I). This result
184 suggests that KDM3A and SAT1 are working in the same pathway in the cellular response to
185 DNA damage.

186 **KDM3A serves as a scaffold to regulate SAT1 alternative splicing**

187 To understand how KDM3A regulates SAT1 alternative splicing, we first asked whether the
188 demethylase activity of KDM3A is critical for its regulation of alternative splicing. To this end,
189 we stably expressed KDM3A with a mutated demethylase domain and silenced endogenous
190 KDM3A in MCF7 cells. We found that overexpression of either WT or the demethylase mutant
191 KDM3A reduced SAT1 exon X inclusion (Fig. 3C and Supplementary Fig. S3J&K). This result
192 indicates that KDM3A's demethylase activity is not essential for alternative splicing
193 regulation and suggests that KDM3A serves as a scaffold to regulate SAT1 alternative
194 splicing. We then silenced the H3K9 methyltransferases G9a and GLP and the H3K9
195 methylations readers: HP1α, HP1β and HP1γ and monitored SAT1 alternative splicing. We

196 observed SAT1 exon X inclusion when G9a or GLP were silenced (Fig. 3D and Supplementary
197 Fig. 3L), similar to the results seen when silencing KDM3A. No change in SAT1 alternative
198 splicing was observed following silencing of H3K9 methylation readers (Supplementary Fig.
199 M&N). This result suggest that it is not the methylation state of H3K9 which regulates SAT1
200 splicing and that the chromatin proteins might serving as a scaffold for splicing factors.

201 **SRSF3 and hnRNPF promote exclusion of SAT1 exon X**

202 We hypothesize that KDM3A serves as a scaffold to recruit a splicing factor to exclude SAT1
203 exon X. Such splicing factors could be those known to act as an adaptor system connecting
204 DNA and histone methylation to the spliceosome SRSF1, SRSF3, SRSF6 and SF3B1[39] and
205 hnRNPF, which was shown to bind KDM3A and regulate alternative splicing of AR variant 7
206 (AR-V7)[25]. We silenced each of these splicing factors and found that both SRSF3 and hnRNPF
207 promote exclusion of SAT1 exon X similar to KDM3A (Fig. 4A and Supplementary Fig.
208 S4A&B). This suggests that SRSF3 and hnRNPF might be working in the same pathway as
209 KDM3A in the regulation of SAT1 alternative splicing.

210 **The SWI/SNF subunit ARID1A promotes SAT1 exon X exclusion**

211 KDM3A phosphorylation on S265 following β-adrenergic stimulation regulates gene
212 expression via the SWI/SNF complex[16]. The SWI/SNF subunit Brm was previously shown to
213 promote exon inclusion of several genes[49]. This led us to ask whether the SWI/SNF complex
214 has a role in SAT1 alternative splicing. To check our hypothesis, we asked whether ARID1A,
215 a SWI/SNF subunit binding to phospho-KDM3A[16], has a role in SAT1 alternative splicing. To
216 this end, we silenced ARID1A and found that it promoted SAT1 exon X exclusion (Fig. 4B and
217 Supplementary Fig. S4C&D). Since ARID1A, KDM3A, and SRSF3 promote SAT1 exon X
218 exclusion, it is possible that they cooperate in the alternative splicing of SAT1 exon X,
219 presumably by forming an adaptor system connecting chromatin to the spliceosome.

220 **KDM3A/SRSF3 adaptor system**

221 The interaction of phospho-KDM3A and ARID1A was described before[16]. In order to test the
222 binding of KDM3A to SRSF3, we immunoprecipitated KDM3A from MCF7 cells stably
223 expressing Flag-KDM3A-V5 using the Flag-tag. Since KDM3A phosphorylation was shown
224 to be important for its binding to ARID1A, we incubated the cells with NCS. The
225 immunoprecipitation demonstrated a physical interaction between KDM3A and SRSF3 (Fig.
226 4C), pointing to the presence of these proteins in the same complex.

227 To check if KDM3A is needed for SRSF3 binding to SAT1 pre-mRNA, we performed RNA-
228 IP for SRSF3 following silencing of KDM3A in MCF7 cells (Supplementary Fig. S4E&F).
229 Monitoring SAT1 pre-mRNA relative to input, we found silencing of KDM3A led to a
230 reduction of approximately 75% in SRSF3 binding to SAT1 pre-mRNA (Fig. 4D). This result
231 supports the hypothesis that the two proteins functionally interact in the regulation of SAT1's
232 alternative splicing.
233

234 **MATERIALS AND METHODS**

235 **Cell culture**

236 MCF7 (ATCC Number: HTB-22) and HEK293T (ATCC Number: CRL-3216) cells were grown
237 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum;
238 cell-lines were maintained at 37°C and 5% CO_2 atmosphere.

239 **RNA interference**

240 A pool of four siRNA oligomers per gene against KDM3A, SRSF1, SRSF3, SRSF6, HP1α, HP1β,
241 HP1γ and SF3B1 were purchased from Dharmacon; SAT1, HNRNPF and ARID1A esiRNA
242 were purchased from Sigma. MCF7 cells were grown to 20–30% confluence and transfected
243 with siRNA using TransIT-X2 transfection reagent following the manufacturer's instructions.
244 After 24 h of incubation, cell culture media was refreshed and then incubated for an additional
245 48-72 h. Knockdown efficiencies were verified by qPCR.

246 **qRT-PCR**

247 RNA was isolated from cells using the GENEzol TriRNA Pure Kit (GeneAid). cDNA synthesis
248 was carried out with the Quanta cDNA Reverse Transcription Kit (QuantaBio). Then, qPCR
249 was performed with the iTaq Supermix (BioRad) on the Biorad iCycler. The comparative Ct
250 method was employed to quantify transcripts, and delta Ct was measured in triplicate.
251 Primers used to amplify the target genes are provided in Supplementary Table 1.

252 **RNA-seq**

253 RNA ScreenTape kit (catalog #5067-5576; Agilent Technologies), D1000 ScreenTape kit
254 (catalog #5067-5582; Agilent Technologies), Qubit RNA HS Assay kit (catalog # Q32852;
255 Invitrogen), and Qubit DNA HS Assay kit (catalog #32854; Invitrogen) were used for each
256 specific step for quality control and quantity determination of RNA and library preparation.

257 For mRNA library preparation: TruSeq RNA Library Prep Kit v2 was used (Illumina). In brief,
258 1 μg was used for the library construction; library was eluted in 20 μL of elution buffer.
259 Libraries were adjusted to 10 mM, and then 10 μL (50%) from each sample was collected and
260 pooled in one tube. Multiplex sample pool (1.5 pM including PhiX 1.5%) was loaded in
261 NextSeq 500/550 High Output v2 kit (75 cycles cartridge and 150 cycles cartridge; Illumina).
262 Run conditions were in paired end (43 × 43 bp and 80 × 80 bp, respectively) and loaded on
263 NextSeq 500 System machine (Illumina). We used rMATS (version3.2.5)[50] to identify
264 differential alternative splicing events between the two sample groups corresponding to all
265 five basic types of alternative splicing patterns. Briefly, rMATS uses a modified version of the
266 generalized linear mixed model to detect differential alternative splicing from RNA-seq data
267 with replicates, while controlling for changes in overall gene expression levels. It accounts for
268 exon-specific sequencing coverage in individual samples as well as variation in exon splicing
269 levels among replicates. For each alternative splicing event, we used the calculation on both
270 the reads mapped to the splice junctions and the reads mapped to the exon body. The results
271 of rMATS analysis gave low numbers of significant AS events with p-value 0.05 and a cut off
272 at FDR < 5%. Thus, one replicate was excluded from the analysis, and then specific parameters
273 were identified manually; with inclusion level difference between groups = 2, and average
274 count >10.

275 **Viral infection**

276 Retroviral or lentiviral particles were produced by expressing KDM3A-P S265 WT or mutants
277 (S265A, S265D)[16] or p-Lenti-V5-KDM3A-WT (addgene #82331) or demethylase MUT
278 (addgene #82332) plasmids respectively in the HEK293T packaging cell-line using TransIT-
279 X2 transfection reagent. MCF7 cells were infected with viral particles and stable integrations
280 were selected.

281 **Immunoblotting and immunoprecipitation**

282 For immunoblotting cells were harvested and lysed with RIPA lysis buffer, and the extracts
283 were run on a 4–12% Bis-Tris gel and transferred onto a polyvinylidene difluoride membrane.
284 For immunoprecipitation cells were washed twice with ice-cold PBS, harvested and lysed for
285 30 min on ice in 0.5% NP40, 150 mM NaCl, 50 Mm Tris pH7.5, and 2 mM MgCl2 supplemented
286 with protease, phosphatase and RNase inhibitors. Supernatants were collected after
287 centrifugation at 21, 000 g for 20 min. Supernatants were immunoprecipitated for 2 h with 40
288 ul of anti-Flag M2 Magnetic Beads (M8823, Sigma Aldrich). Beads were washed sequentially
289 for 5 min. Beads were boiled in sample buffer and loaded onto the gel for analysis. The

290 samples were subjected to standard immunoblotting analysis using polyvinylidene difluoride
291 membranes and enhanced chemiluminescence.

292 **Flow cytometry**

293 Cells were trypsinized, washed with PBS, fixed overnight at -20°C with 70% ethanol in PBS,
294 washed with PBS, and left for 30 min at 4°C. The cell suspension was then incubated with PBS
295 containing 5 μg/ml DNase-free RNase and stained with propidium iodide (PI). Data was
296 acquired using LSRII Fortessa analyser machine at 10,000 events/sample. The percentage of
297 cells in each cell cycle phase was determined using Flow Jo software.

298 **Chemical reagents**

299 Neocarzinostatin (NCS) was obtained from Sigma (Ca# 067M4060V). PKA inhibitor was
300 obtained from Sigma (P6062).

301 **RNA-immunoprecipitation (RNA-IP)**

302 Cells were washed with ice-cold PBS, harvested, and lysed for 30 min on ice in a buffer
303 containing 1% NP40 150 mM NaCl, 50 mM Tris pH = 7.5, and 1 mM EDTA supplemented
304 with protease and RNAsin inhibitors followed by sonication in an ultrasonic bath (Qsonica,
305 Q800R2 Sonicator) for 6 cycles of 5 s ON and 30 s OFF. Supernatants were collected after
306 centrifugation at 21,000 g for 20 min. Antibody was added for 2 h at 4°C. Protein A and G
307 sepharose beads were added for an additional 1 h. Beads were washed 4 times and GeneZol
308 added for RNA extraction. Serial dilutions of the 10% input cDNA (1:5, 1:25, 1:125) were
309 analyzed by SYBR-Green real-time qPCR. The oligonucleotide sequences used are listed in
310 Supplementary Table S1.

311

312 **DISCUSSION**

313 Chromatin structure is constantly changing in response to the cell's environment. These
314 dynamic alterations are the basis of a changing cellular transcriptome that in turn adjust the
315 cell to the new environment. Here we suggest a transcriptome change by alternative splicing
316 giving rise to isoforms that are better able to respond to the environment. To this end we
317 studied the role of the signal-sensing scaffold KDM3A. While KDM3A's role in temperature
318 sensing is well established, we have now found that it is phosphorylated following DNA
319 damage to regulate alternative splicing, as well as the expression of cell cycle genes.

320 DNA damage has been shown multiple times by us and others to regulate the transcriptome
321 and specifically alternative splicing[51-54]. Here we add that the mediator of the alternative

322 splicing regulation can be chromatin proteins that are a known part of the massive cellular
323 response to DNA damage. Splicing factors were also demonstrated to be part of the signal
324 transduction following DNA damage. In particular SRSF3 was demonstrated to serve as a
325 regulator of genome integrity and cell cycle[55-58]. Our work demonstrates that chromatin
326 alternations following DNA damage can serve as a scaffold to recruit SRSF3 to specific cell
327 cycle genes. This observation adds a level of regulation to splicing factors' preference for their
328 targets following a cellular environment change.

329 Histone modifications over alternatively spliced gene regions have previously been shown to
330 act as recognition sites for epigenetic adaptor proteins, which in turn recruit splicing
331 factors[4,30]. Here we demonstrate that phosphorylated S265 KDM3A itself serves as a scaffold
332 for splicing factor recruitment with the SWI/SNF subunit ARID1A. Another subunit of the
333 SWI/SNF complex, Brm, was indicated to regulate alternative splicing by promoting
334 inclusion of exons via slowing down of RNA polymerase II elongation[49]. However, our
335 results show that ARID1A promotes exclusion of SAT1 exon X. This suggest that the
336 mechanism of the SWI/SNF complex on the SAT1 gene is different to that described before.

337 Our results demonstrate that the H3K9me2 methyltransferases, G9a and GLP, and its
338 demethylase, KDM3A, both promote exclusion of SAT1 exon X. This result is of special
339 interest and provide strong evidence that KDM3A serves as the scaffold, and not the H3K9
340 modification, for recruiting SRSF3. Our previous work found HP1γ to bind H3K9me2 and
341 recruit the splicing factor SRSF1[30]. This model suggests that KDM3A enzymatic activity will
342 abolish this adaptor system and will promote the opposite alternative splicing outcome.
343 However, SAT1 is a proof that G9a and KDM3A can work in a similar way to regulate
344 alternative splicing. A deeper investigation is needed to learn the fine tuning of each of these
345 chromatin factors.

346 Demethylation of H3K9 by KDM3A was shown to promote alternative splicing of AR7 by
347 recruiting hnRNPF[25]. This work describes a mechanism that is distinct from the one we
348 describe here as it requires KDM3A's enzymatic activity and our work shows that the
349 methylase mutant of KDM3A can also serve as an alternative splicing regulator. Our results
350 shed light on the many mechanisms by which chromatin can regulate alternative splicing.

351

352 **DATA AVAILABILITY**

353 The RNA-seq data has been deposited to the Gene Expression Omnibus (GEO) with the
354 dataset identifier GSE141948.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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Figure Legends

Figure 1. **KDM3A is a regulator of transcription and alternative splicing of cell cycle and DNA damage genes.** MCF7 cells were transfected with non-targeting siRNA (siNT) and siKDM3A for 72 h. RNA was extracted, libraries prepared and RNA-seq was conducted. **A**. Venn diagram representing differentially expressed genes (blue) and alternatively spliced genes (red). **B & C**. Functional analysis was conducted using the DAVID functional

538 annotation tool (DAVID, https://david.ncifcrf.gov/) for (**B**) differentially expressed genes
539 and (**C**) alternative splicing genes.

540 Figure 2. **KDM3A is phosphorylated by PKA following double strand breaks. A**. MCF7 cells
541 treated with 200 ng/ml NCS for 1 h. Immunoblotting was conducted with the indicated
542 antibodies. **B**. MCF7 cells were treated with PKAi for 30 min following treatment with 50 and
543 200 ng/ml NCS for 1 h. Immunoblotting was conducted with the indicated antibodies. **C**.
544 MCF7 cells stably expressing either wild-type KDM3A or KDM3A mutated at serine 265 to
545 alanine (S265A) or to aspartic acid (S265D) were incubated with 200 ng/ml NCS, and analyzed
546 8 h later using flow cytometry. The amount of cells accumulated in S-phase is shown as a fold-
547 change relative to untreated cells. **D**. MCF7 cells were incubated with PKAi for 30 min
548 followed by treatment with 200 ng/ml NCS, and analyzed 8 h later using flow cytometry. The
549 amount of cells accumulated in S-phase is shown as a fold-change relative to untreated cells.
550 C and D, plot represents the mean of four independent experiments and ±SD (* $p<0.05$; **
551 $p<0.01$).

552 Figure 3. **KDM3A regulates SAT1 alternative splicing independent of its demethylase**
553 **enzymatic activity. A**. Schematic representation of SAT1 alternative splicing. Rectangles:
554 exons, black lines: introns. **B**. MCF7 cells were transfected with non-targeting siRNA (siNT)
555 and siSAT1 for 72 h, followed by treatment with 200 ng/ml NCS, and analyzed 8 h later using
556 flow cytometry. The amount of cells accumulated in S-phase is shown as a fold-change
557 relative to untreated cells. **C**. MCF7 cells stably expressing either the empty vector, wild-type
558 KDM3A or KDM3A mutated demethylase (mut). Total RNA was extracted and analyzed by
559 real-time PCR for SAT1 exon X inclusion. PSI was calculated by SAT1 exon X relative to SAT1
560 total mRNA amount. **D**. MCF7 cells were transfected with non-targeting siRNA (siNT) and
561 siG9a or siGLP for 72 h. Total RNA was extracted and analyzed by real-time PCR for SAT1
562 exon X inclusion. PSI was calculated by SAT1 exon X relative to SAT1 total mRNA amount.
563 Values represent averages of three independent experiments ±SD (* $p<0.05$; ** $p<0.01$; ***
564 $p<0.001$; **** $p<0.0001$).

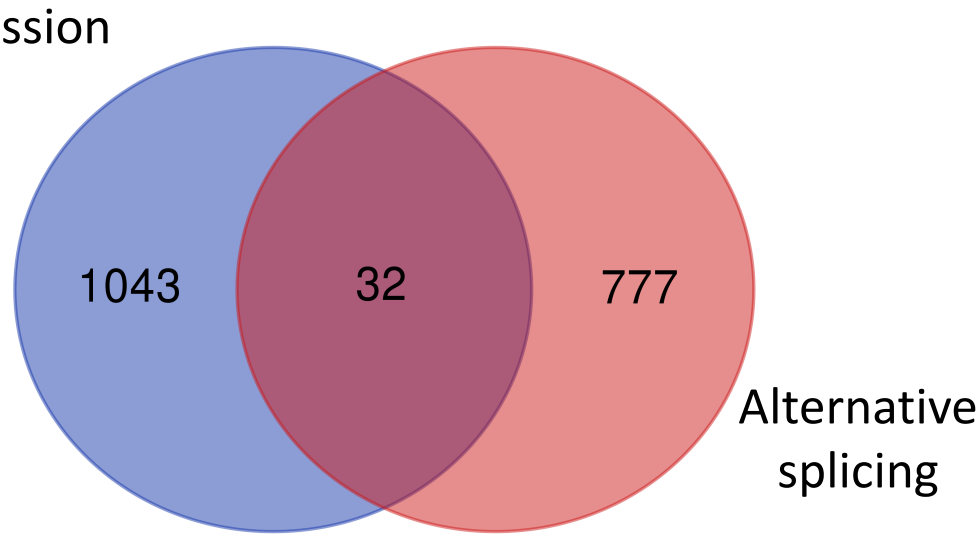
565 Figure 4. **KDM3A allows for SRSF3 binding to SAT1 pre-mRNA. A**. MCF7 cells were
566 transfected with non-targeting siRNA (siNT) or siSF3B1, siSRSF1/3/6 and sihnRNPF for 72
567 h. Total RNA was extracted and analyzed by real-time PCR for SAT1 exon X inclusion. PSI
568 was calculated by SAT1 exon X relative to SAT1 total mRNA amount. **B**. MCF7 cells were
569 transfected with non-targeting siRNA (siNT) or siARID1A for 72 h. Total RNA was extracted
570 and analyzed by real-time PCR for SAT1 exon X inclusion. PSI was calculated by SAT1 exon
571 X relative to SAT1 total mRNA amount. **C**. MCF7 cells stably expressing FLAG-KDM3A-V5

572 were treated with 200ng/ml NCS for half an hour. KDM3A was immunoprecipitated using
573 the Flag tag, and detected with V5 (for KDM3A) and SRSF3. * long exposure. **D**. RNA-IP of
574 SRSF3 in MCF7 cells that were transfected with non-targeting siRNA (siNT) or siKDM3A for
575 72 h. Real-time PCR analysis of SAT1 intron 3 (near exon X) relative to input. Values represent
576 averages of three independent experiments ±SD (* $p<0.05$; ** $p<0.01$; **** $p<0.0001$).

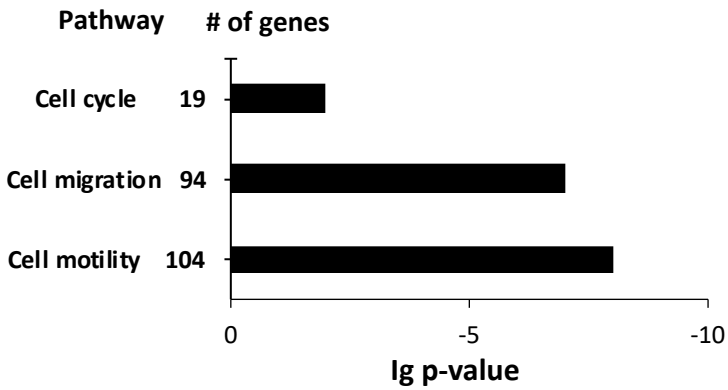
577

Fig 1.

A


Expression
1043
32
777
Alternative splicing

B


Pathway
# of genes
Cell cycle 19
Cell migration 94
Cell motility 104
0
-5
-10
lg p-value

C

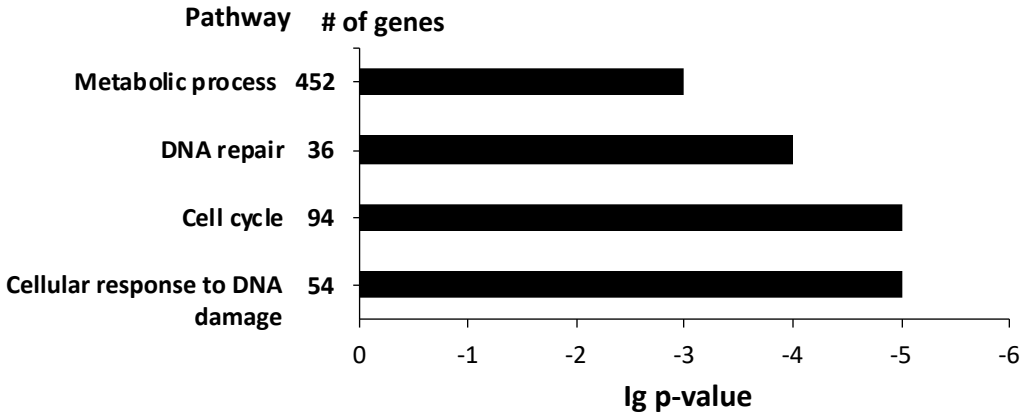

Pathway
# of genes
Metabolic process 452
DNA repair 36
Cell cycle 94
Cellular response to DNA damage 54
0
-1
-2
-3
-4
-5
-6
lg p-value

Fig 2.

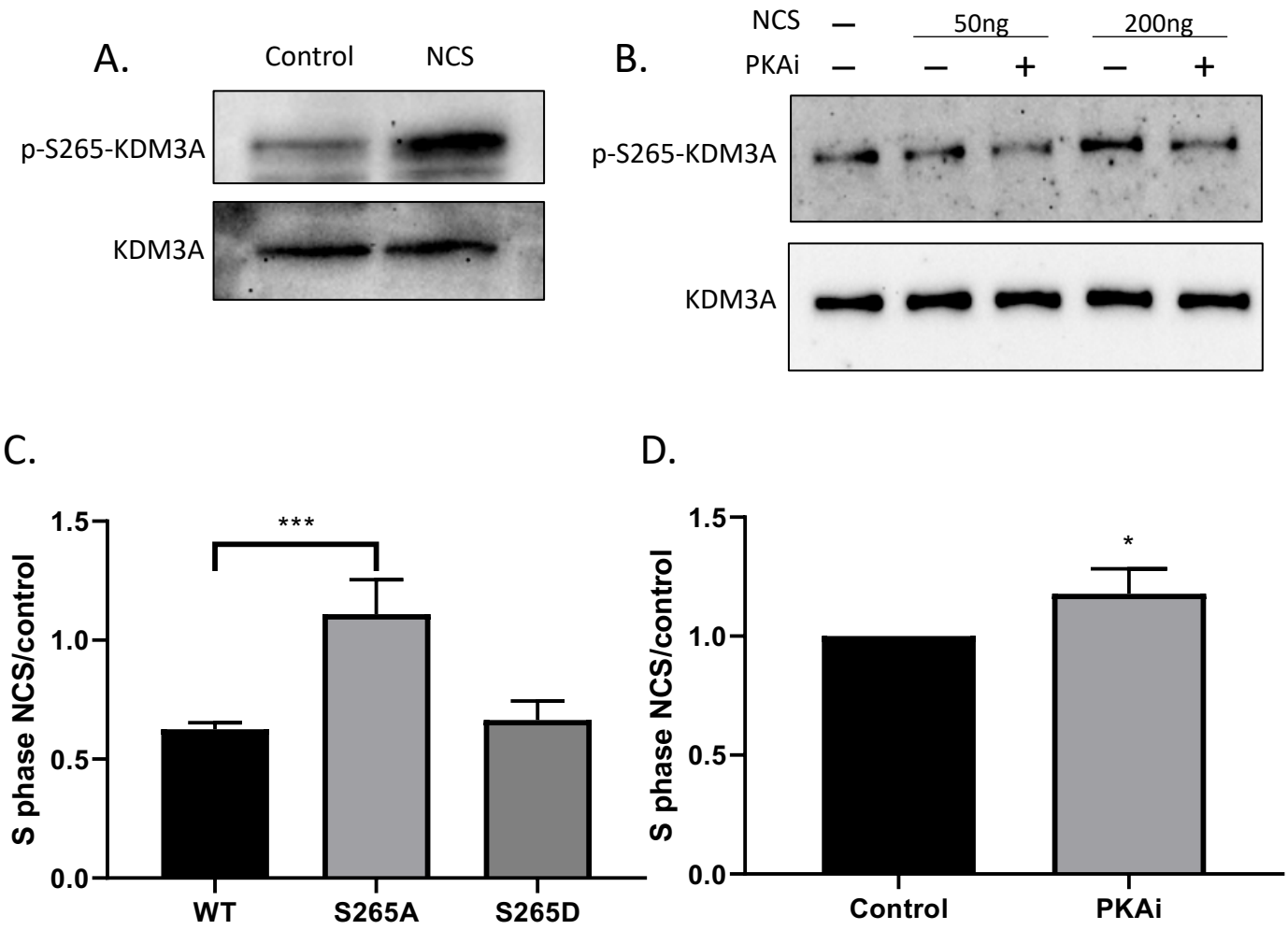
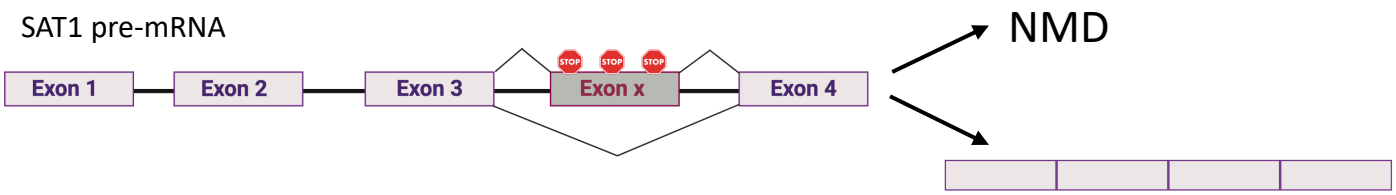
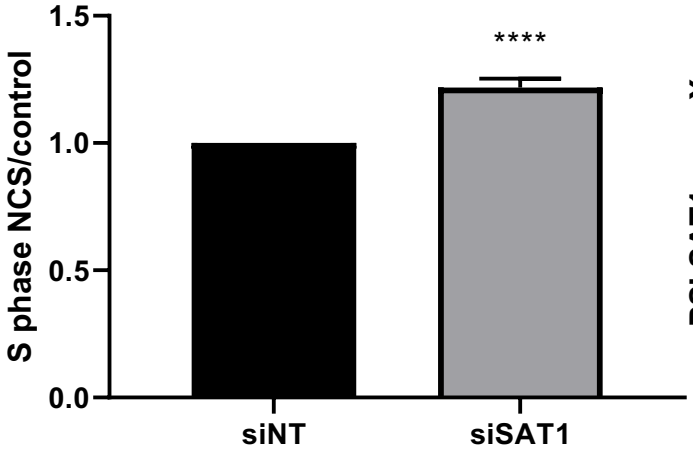

A.
Control
NCS
p-S265-KDM3A
KDM3A
B.
NCS
50ng
200ng
PKAi
p-S265-KDM3A
KDM3A
C.
S phase NCS/control
1.5
1.0
0.5
0.0
***
WT
S265A
S265D
D.
S phase NCS/control
1.5
1.0
0.5
0.0
*
Control
PKAi

Fig 3.

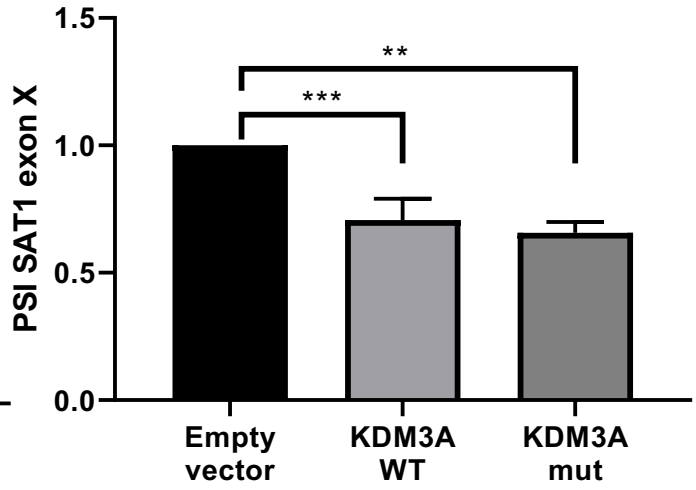
A.


SAT1 pre-mRNA
Exon 1
Exon 2
Exon 3
Exon x
Exon 4
NMD

B.


S phase NCS/control
1.5
1.0
0.5
0.0
****
siNT
siSAT1

C.


PSI SAT1 exon X
1.5
1.0
0.5
0.0
**
***
Empty vector
KDM3A WT
KDM3A mut

D.

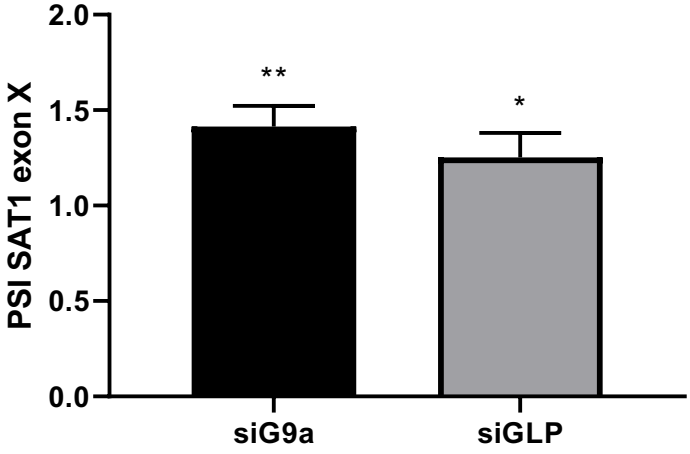
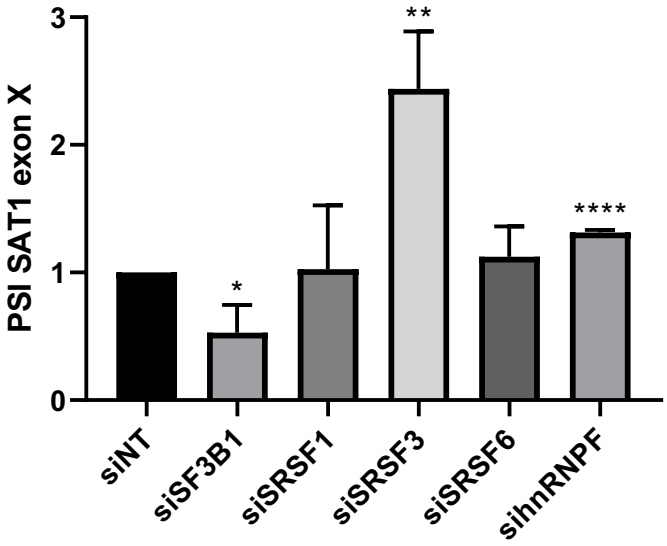
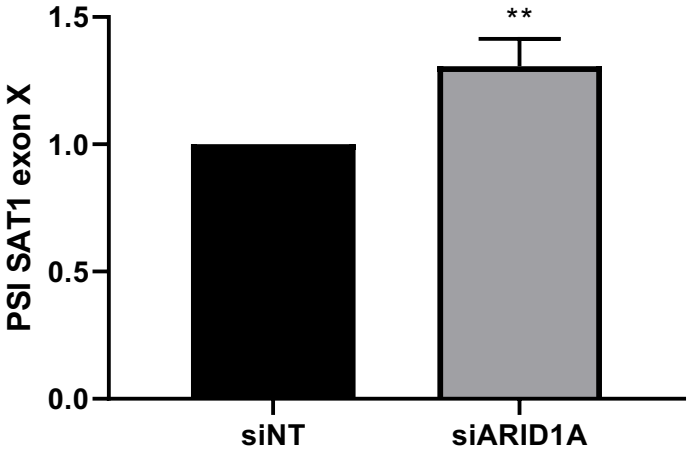

PSI SAT1 exon X
2.0
1.5
1.0
0.5
0.0
**
*
siG9a
siGLP

Fig 4.

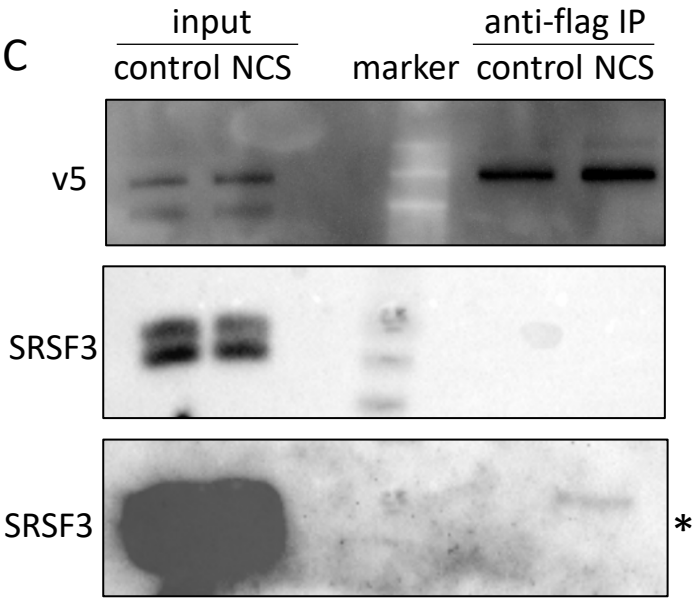
A


PSI SAT1 exon X
3
2
1
0
*
**
****
siNT
siSF3B1
siSRSF1
siSRSF3
siSRSF6
sihnRNPF

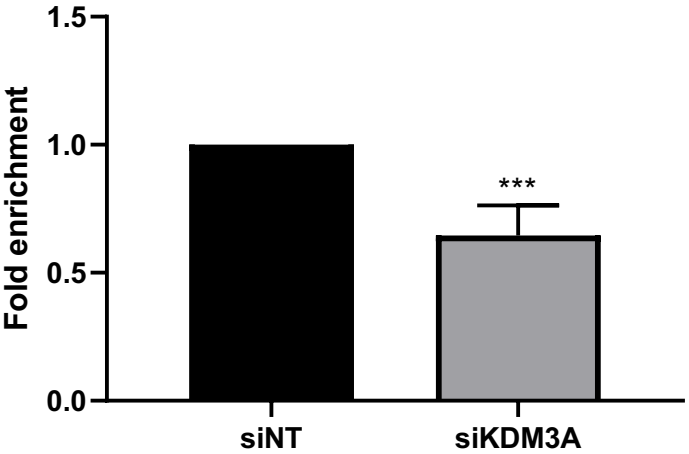
B


PSI SAT1 exon X
1.5
1.0
0.5
0.0
**
siNT
siARID1A

C


input
control NCS
marker
anti-flag IP
control NCS
v5
SRSF3
SRSF3
*

D


Fold enrichment
1.5
1.0
0.5
0.0
***
siNT
siKDM3A